行政院國家科學委員會專題研究計畫 成果報告

台灣本土五葉松萃取物免疫功能評估及促進其商品產業化研究成果報告(精簡版)

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（計畫名稱）

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中　華　民　國　99 年　09 月　10 日

前言（Introduction）

松的介紹

松樹在民間療法與預防疾病的應用已經進行多年，但相關的科學研究卻不多，尤其在台灣本土松類相關之研究更屈指可數。台灣的島嶼地形分佈著許多高山，其中蘊藏著豐富的高山植物，其中松為一大宗，而松的木材不僅在建築材料上具有重要經濟價值，而且是許多藥理活性物質的來源。在中國傳統典籍中藥大辭典中記載，松樹不同的部位具有不同的療效，如松香的功用在祛風、風濕痹痛、排膿、拔毒等；松毬的功用在治風痹、補氣、痔疾等；松葉具有治療失眠、殺蟲、止癢；而松樹根可治療風濕性關節炎。根據另一典籍神農本草經記載：「松葉藥效，促進毛髮生長、定五臟、止饑、延壽，以水及麵飲服之或擣屑丸服，可治惡疾」。而根據日本漢方協會研究也指出，松葉具有毛髮生長、安養五臟、整腸健胃、延年益壽、防止齲齒、治療腦中風、去除血液中膽固醇、防止動脈硬化、防止老化、出血、老人癡呆症、高血壓、氣喘、神經痛、關節炎、沙啞、恢復聲音等保健功能。松葉提煉之精油，在去除血中膽固醇、減少血液黏度等方面具有特定功效。而松葉之新芽可有效防止中風、促進血液安定、防止腦中風之麻痺及治癒語言障礙。在日本某些醫院則早已應用松葉於診治高血壓、中風、腦溢血、動脈硬化、糖尿病、氣喘、過敏性鼻炎、痛風、肺結核、神經痛、牙齦腫脹、牙周病等相關疾病。

台灣五葉松（*Pinus morrisonicola* Hayata）的介紹

台灣五葉松（*Pinus morrisonicola* Hayata）又稱台灣白松（Taiwan white pine），屬於裸子植物中之松科白松亞屬，針葉五根一束，長約五至九公分，種子有刺，外型為大喬木，毬果為長型長約七至十公分，種鱗之鱗盾成菱形，種子有翅，松樹的樹皮在樹齡屆二十二年後開始出現龜甲狀之裂紋，越老越明顯，分佈於中央山脈海拔三百至兩千公尺之中低海拔山區（如在台北石碇皇帝殿山頂稜線上，中部地區之谷關、新社、大坑，以及埔里分佈較廣）。

松樹（屬）之植物純化成份

由目前文獻得知，自松樹（屬）植物純化而得的植物多酚類已知有來自松屬（*Pinus spp.*）中萃取而得的多酚成份 Pinostrobin, Ampelopsin, Pinobanksin, Cryptostrobin, Pinocembrin, Tectochrysin, Strobopinin；自五葉松（*Pinus morrisonicola*）中萃取 Chrysin, Izalpinin, 3,5,7-Trihydroxy-6-methylflavanone 3-acetate；自美國黃松（*Pinus ponderosa*）中萃取 Pinomyricetin, Pinoquercetin；自北美短葉松（*Pinus banksiana*）中萃取 Helichrysoside；自傑弗裏松、約弗松、黑材松（*Pinus jeffreyi*）中萃取 Kaempferol 3-(6”-ferulylglucoside), Laricitrin 3-glucoside, Syringetin 3-glucoside；自北美白松（*Pinus strobes*）中萃取 Strobochrysin；自長白松（*Pinus sylvestris*）中萃取 Isorhamnetin 3-(6”-acetylgalactoside), Kaempferol 3-(3”,6”-di-p-coumaryl-glucoside), Quercetin 3-(6”-acetylgalactoside), Quercetin3-(3”-p-coumarylglucoside), Quercetin3-(3”,6”-di-pcoumarylglucoside), Sylpin, Trifolin；自美國黑松、扭葉松、小杆松（*Pinus contorta*）中萃取 Isorhamnetin 3-(6”-acetylglucoside), 6-C-Methylkaempferol, 6-C-Methylkaempferol 3-glucoside, Quercetin 3,3’-doglucoside, Tiliroside；自馬尾松（*Pinus massoniana*）中萃取 Eriodictyol 3’-o-glucoside, (2R,3R)-Taxifolin 3-glucoside, Taxifolin 3’-(6”-phenylacetylglucoside), 3,5,7,4’-Tetrahydroxy-6-methylflavanone 7-glucoside；自沙松（*Pinus clausa*）中萃取 Alpinone；自蘇格蘭松、長白松、歐洲赤松（*Pinus sylvestris*）中萃取 Ampelopsin 3’-glucoside, Epigallocatechin-(4β→8)-catechin, Procyanidin B6, Taxifolin 3’-glucoside；自赤松（*Pinus densiflora*）中萃取 Isoglucodistylin, Taxillusin；自喬松（*Pinus excelsa*）中萃取 Flavokawin B；自糖松（*Pinus lambertiana*）中萃取 3,5,7-Trihydroxy-6-methylflavanone；自輻射松（*Pinus radiata*）中萃取 Procyanidin B5, Procyanidin B6, Procyanidin C2；自華山松（*Pinus armandii*）中萃取(2R,3R)-Pinobanksin 3-acetate；自北美喬松（*Pinus strobes*）中萃取 3,5,7-Trihydroxy-6-methylflavanone；和自火炬松（*Pinus taeda*）中萃取 Arecatannin A1, Arecatannin B1, Epicatechin-(4β→6)-epicatechin-(4β→8)-catechin, Procyanidin B5, Procyanidin B7,

Procyanidin C (Jeffrey et al., 1999)。

松萃取物抑制腫瘤及其生物功能之研究

先前研究針對日本五葉松（*P. parvilofora*）成份分析指出其含有苯多酚化合物、葉綠素、烯類、黃酮類、維生素 A、D、K、精油、松香酸、松油帖、甲狀腺素、各類胺基酸、碳氫化合物及不飽和脂肪酸等。分析松針萃取物成分，具有類固醇皂苷（steroidal saponins）、triterpenoidal saponins、萜烯（terpenes）和芳香族酸（aromatic acid）等有效抗癌物質 (Kong et al., 1995)。而另一學者研究推測松針中可能具有效抗癌成分為原色花青素（proanthocyanindins）成分 (Nyman, 1985)。花青素屬於類黃酮化合物（flavonoids）的天然色素，而許多研究發現植物成分中的類黃酮化合物具有抗腫瘤活性，如 Semecarpus anacerdium L.的果實萃取物其成分含類黃酮化合物，其對由黃麴毒素（aflatoxin B1）誘導產生的肝細胞癌有抑制作用 (Premalatha and Sachdanandam, 1999)。由紅葡萄所釀製的紅酒含有豐富的類黃酮化合物，有槲皮酮（quercetin）、兒茶素（catechin）、白藜蘆醇（Resveratrol） 及五倍子酸（gallic acid），學者利用此些成分餵食帶有皮膚腫瘤的老鼠，結果均可抑制皮膚腫瘤生長 (Soleas et al., 2002)。依據松屬相關文獻記載松針具有抗致突變性及抗腫瘤效果 (Kong et al., 1995)，其成份含有 Proanthocyanidins (Nyman, 1985)；毬果具有抑制老鼠乳腺腫瘤生長、抗菌等功效，其成份含有和醣類鍵結之木質素類似物質及 Proanthocyanidin (Eberhardt and Young, 1994; Sakagami et al., 1991)；樹皮具有抗氧化、抗發炎、抑制血小板凝集等功效 (Bito et al., 2000; Cheung et al., 1994; Packer et al., 1999)，其成份是多酚類 Procyanidins 及其它類黃酮素 (Cheynier et al., 1999)、diterpene acids（如 levopimaric acid ）(Cheung et al., 1994)。

台灣五葉松及其生物活性

Hsu et al., (2005)等人研究指出台灣五葉松松針（*Pinus morrisonicola* Hay.）可清除超氧化物並抑制白血病細胞 U937 的生長。而另一研究團隊也發現台灣五葉松松針（*Pinus morrisonicola* Hay.）在 low-density lipoprotein（LDL）的氧化和巨噬細胞一氧化氮產生的保護效應就類似一些已被研究發現具有生物活性的有效成分，而台灣五葉松對 LDL 的抗氧化效應和降低發炎部位產生過多的一氧化氮也已被確認 (Yen et al., 2008)。除了以上研究之外，對於台灣五葉松萃取物的生物活性效應至今尚未被明確闡明。

白血病簡介

現今國人深陷於癌症疾病之苦，四至五人中就有一人死於癌症，許多家庭同時面臨生離死別的痛苦。而白血病又位於癌症死亡率排名前十名，據統計，全世界每十萬人就有 2 至 9 人罹患白血病，從嬰兒至老年人皆有。白血病是不正常的白血球大量增生所造成的，常見症狀包括貧血、易出血、淋巴結腫大、肝脾腫大、體重減輕等。白血病的病因較為複雜，可能致病因子為: 化學藥劑傷害、輻射線汙染、自體免疫功能缺陷、病毒感染、基因突變及自身體質因素等所造成 (陳來登等翻譯，2002)。以下是四種是最常見的白血病：[1] 急性淋巴性白血病（ALL）是常見於年幼小孩的一類白血病，但此疾病同樣會影響成人，特別是 65 歲以上的年長者。 [2] 急性骨髓性白血病（AML）在成人和孩童都會發生此類的白血病，有時亦被稱為急性非淋巴性白血病（ANLL）。 [3] 慢性淋巴性白血病（CLL）常發生於 55 歲以上的成人。但有時發生於年輕的成人，幾乎不發生在小孩身上。 [4] 慢性骨髓性白血病（CML）主要是發生於成人，少部分的小孩也會同樣產生此類疾病。而本研究所使用的 human acute promyelocytic leukemia cells (HL-60 cells)屬於急性骨髓性白血病（AML）。

細胞週期

細胞週期是指能持續分裂的正常細胞從一次有絲分裂結束後繼續生長，再到下一次分裂結束前的一個循環過程。整個週期表示為：G1 期→S 期→G2 期→M 期，而細胞週期主要區分為兩個時期，一為分裂期（mitotic phase），一為分裂間期（interphase）。間期又可再分為 G1（gap 1）期、S（synthesis）期、G2（gap 2）期。G1 期為 M 期結束與 DNA 複製間的一段空隙，S 期為合成期，染色體 DNA 在

此期間複製；G2 期為細胞分裂前期，是細胞進入有絲分裂之前的準備期。在這段期間，DNA 的複製受到嚴密監控，以確保數量到達雙倍，同時細胞成長並完成內容物複製。正常細胞週期的進行可維持細胞增殖及死亡間的平衡，如果失去平衡，就可能發展為腫瘤等相關疾病。目前所知調控細胞週期的機制非常複雜，已知的調控因子主要包含 cyclin、CDK（cyclin dependent kinase）; cyclin 會和 CDK 結合並活化 CDK。被分離出來的 cyclin 包括 cyclin A、B1、B2、D1、D2、D3、E、H 等；不同的 cyclin 會和不同的 CDK（如 CDK2、CDK4、CDK5、CDK6 等）結合，各種 cyclin 的量會隨細胞週期的進行而做週期性的改變 (O'Connor et al., 2000)。在細胞週期運轉的過程中，不同的 cyclin 會和特定的 CDK 結合。G1:細胞受生長因子刺激離開 G0 期，首先出現的是 cyclin D，其與 CDK4、CDK6 結合，將 Rb（retinoblastoma protein）磷酸化後，使與 E2F 分開，故 E2F 可促進與 DNA 複製相關基因的表現。而在 G1 後期，cyclin E 增加，和 CDK2 結合而使細胞進入 S 期。在 S 期時，cyclin E 消失，CDK2 與 cyclin A 結合;G2/M 期時，CDK-activating kinase（CAK）作用於 CDK1（CDC2），使其磷酸化; 而 CDC25（phophatase）則會使 CDK1 去磷酸化進而活化 CDK1，活化的 CDK1 會和 cyclin A 與 cyclin B 結合，以調節有絲分裂之進行（黃雯雯，2002）。

細胞凋亡

細胞凋亡，是一種計畫性的細胞死亡，具有特定的形態和生化變化，包括了細胞皺縮，染色質濃縮，核內DNA片段化，細胞膜泡狀突起，且細胞會破碎分解為凋亡小體 (Platt et al., 1998)。細胞凋亡透過兩個主要路徑所活化，一是透過粒線體傳導路徑（內在傳導路徑），而另一則是凋亡訊息受體傳導路徑（外在傳導路徑） (Thornberry et al., 1997)。 Cytochrome *c*是一個計劃性細胞死亡的重要調節者。Cory和Adams, (2002)的研究指出cytochrome *c*從粒線體釋放出到細胞質可藉由Bcl-2蛋白家族成員所調控。Bcl-2蛋白家族成員在細胞凋亡過程中扮演著重要的角色，其中包含了抗細胞凋亡成員（Bcl-2）和促細胞凋亡成員（Bax, Bad, and Bak）(Hunt and Evan, 2001)。Caspase-3會在細胞凋亡後期藉由各種刺激的誘導所活化 (Thornberry and Lazebnik, 1998)。Green和Reed, (1998)證實在細胞凋亡的早期粒線體膜電位會降低，是因為caspase-3/caspase-9活化後促使cytochrome *c* 從粒線體釋放出到細胞質。

研究動機與目的

除了先前提到的研究之外，對於台灣五葉松萃取物的生物活性效應及其明確的影響細胞生理機制至今尚未被完全了解，近年在亞洲民間常使用新鮮五葉松葉製成果汁飲用 (Kim and Chung, 2000)，或以樹皮、松葉泡酒、製成茶包來預防疾病或防止身體痠痛。在先前研究文獻指出松萃取物的許多生物活性且具有治療、預防疾病效應，其中也包括了癌症的治療。因此我們認為台灣五葉松萃取物也和其他松萃取物一樣具有類似的治療效用，故本研究想深入了解台灣五葉松萃取物抑制癌細胞的增生與促進細胞凋亡之影響，並找出台灣五葉松萃取物中有效的活性成份，以期未來可提供新的療法或成為癌症治療的輔助劑。

結果（Results）

Effects of *Pinus morrisonicola* Hayata extract and its compounds on cell population growth

The inhibitory effects of *Pinus morrisonicola* Hayata extract on the cell population growth of human acute promyelocytic leukemia cells (HL-60 cells), acute T leukemia Jurkat cells (JK cells), and peripheral blood mononuclear cells (PBMCs) were treated with different concentrations (5 μg/ml, 50 μg/ml, and 500 μg/ml) of *Pinus morrisonicola* Hayata extract. Cell viability was measured after 0, 3, 6, 12, 24 hours by trypan blue exclusion (Fig. 1). We have observed > 80 % cell death in HL-60 and JK cells treated with 500 μg/ml *Pinus morrisonicola* Hayata extract. On the other hand, PBMCs did not display a significant cytotoxicity even after 24 hours of incubation with *Pinus morrisonicola* Hayata extract.

The cell cycle effect of *Pinus morrisonicola* Hayata extract treated in HL-60 cells

The addition of *Pinus morrisonicola* Hayata extract to HL-60 cells possibly has the effects on cell cycle.

We examined the effect of *Pinus morrisonicola* Hayata extract in the regulation of cell cycle. After being treated with *Pinus morrisonicola* Hayata extract for the indicated time. G1 phase proteins were detected by immunoblotting with cyclin E, D, CDK2 and CDK4 antibodies, or the S and G2/M phase proteins with cyclin A, B and CDK1 antibodies. The results revealed that decreased the expression of cyclin D and E after the treatment of *Pinus morrisonicola* Hayata extract (Fig. 2A). By the experiments of flow cytometry with PI staining, Figure 2B showed the percentage in the G1 phase was increased at 12 h after *Pinus morrisonicola* Hayata extract addition, in opposition to S and G2/M phase. These findings were consistent with G1 arrest.

***Pinus morrisonicola* Hayata extract induces apoptotic cell death**

On the basis of the results in Figure 1, HL-60 cells were selected for the study of the induction of *Pinus morrisonicola* Hayata extract on cell apoptosis. The results indicated that *Pinus morrisonicola* Hayata extract decreased the number of HL-60 cells in a time- and dose-dependent manner. Moreover, *Pinus morrisonicola* Hayata extract did not affect the survival of normal peripheral blood mononuclear cells (Fig. 1C). Figure 3 shows the effect of *Pinus morrisonicola* Hayata extract induced apoptosis in HL-60 cells. The nuclear morphology of untreated and treated cells is shown in Figure 3A using acridine orange staining, respectively. Acridine orange staining showed apoptotic bodies when cells were treated with 500 μg/ml *Pinus morrisonicola* Hayata extract for 24 h. Cells undergoing apoptosis revealed a characteristic cleavage of DNA into oligonucleosome fragments manifesting as DNA laddering, a hallmark of apoptosis. The addition of *Pinus morrisonicola* Hayata extract to HL-60 cells resulted in the appearance of internucleosomal DNA ladder (Fig. 3B). The addition of *Pinus morrisonicola* Hayata extract to HL-60 cells resulted in a marked increase in the level of accumulation of the sub-G1 phase (apoptotic cells) (Fig. 3C).

***Pinus morrisonicola* Hayata extract induces apoptosis via intrinsically mediated pathway**

The effects of *Pinus morrisonicola* Hayata extract on the protein expression of Bax, Bcl-2, caspase-9, caspase-3, and PARP in HL-60 cells are shown in Figure 4. The level of pro-apoptotic protein expression of Bax was increased in a time-dependent manner, in comparison to the control after treatment with 500 μg/ml *Pinus morrisonicola* Hayata extract for 6 h. *Pinus morrisonicola* Hayata extract treatment at 500 μg/ml for 18 h has no significantly decreased of Bcl-2 (antiapoptotic protein) expression in comparison to the control. Moreover, the results showed that exposure of HL-60 cells to *Pinus morrisonicola* Hayata extract (500 μg/ml, 6 h) caused the degradation of pro-caspase-9 and pro-caspase-3, which generated a fragment of caspase-9 and caspase-3. *Pinus morrisonicola* Hayata extract treatment at 500 μg/ml for 6 h significantly increased the degradation of PARP in comparison to the control.

Effects of water-phase partition from *Pinus morrisonicola* Hayata extract induce apoptotic cell death

The results indicated that *Pinus morrisonicola* Hayata extract partition of water phase decreased the number of HL-60 cells in a time- and dose-dependent manner. Figure 5 shows the effect of *Pinus morrisonicola* Hayata extract partition of water phase induced apoptosis in HL-60 cells. Cell viability was measured after 0, 3, 6, 12, 24 hours by trypan blue exclusion (Fig. 5A). The nuclear morphology of untreated and treated cells is shown in Figure 5B using acridine orange staining, respectively. Acridine orange staining showed apoptotic bodies when cells were treated with 500 μg/ml *Pinus morrisonicola* Hayata extract partition of water phase for 24 h. Cells undergoing apoptosis revealed a characteristic cleavage of DNA into oligonucleosome fragments manifesting as DNA laddering, a hallmark of apoptosis. The addition of *Pinus morrisonicola* Hayata extract partition of water phase to HL-60 cells resulted in the appearance of internucleosomal DNA ladder (Fig. 5C).

Effects of compounds from *Pinus morrisonicola* Hayata extract on cell population growth

The high-performance liquid chromatography (HPLC) profile indicated that *Pinus morrisonicola* Hayata extract contains pinocembrin, tiliroside, and chrysin (Fig. 6A, B). And the MS-MS chromatograms also confirmed it (Fig. 6C). In this study, the effects of dihydromyricetin, pinocembrin, tiliroside, and chrysin on the cell viability of HL-60 cells are shown in Figure 7. The results showed that the addition of dihydromyricetin, pinocembrin, tiliroside, and chrysin to the growth medium decreased the rate of cell population growth of HL-60 cells.

Chrysin induces apoptotic cell death

The results indicated that chrysin decreased the number of HL-60 cells in a dose-dependent manner. Figure 8 shows the effect of chrysin induced apoptosis in HL-60 cells. The nuclear morphology of untreated and treated cells is shown in Figure 8A using acridine orange staining, respectively. Acridine orange staining showed apoptotic bodies when cells were treated with 200 μM chrysin for 48 h. Cells undergoing apoptosis revealed a characteristic cleavage of DNA into oligonucleosome fragments manifesting as DNA laddering, a hallmark of apoptosis. The addition of chrysin to HL-60 cells resulted in the appearance of internucleosomal DNA ladder (Fig. 8B).

Chrysin induces apoptosis via intrinsically mediated pathway

The effects of chrysin on the protein expression of caspase-9, caspase-3, and PARP in HL-60 cells are shown in Figure 9. The results showed that exposure of HL-60 cells to chrysin (100 μM, 6 h) caused the degradation of pro-caspase-3, which generated a fragment of caspase-3. Chrysin treatment at 100 μM for 6 h significantly increased the degradation of PARP in comparison to the control.

The cell cycle effect of chrysin treated in HL-60 cells

The addition of chrysin to HL-60 cells possibly has the effects on cell cycle. We examined the effect of chrysin in the regulation of cell cycle. After being treated with chrysin for the indicated time. G1 phase proteins were detected by immunoblotting with cyclin E and cyclin D antibodies, or the S and G2/M phase proteins with cyclin A and cyclin B antibodies. The results indicated that decreased the expression of cyclin D after the treatment of chrysin (Fig. 10). This finding was consistent with G1 arrest.

Dihydromyricetin induces apoptotic cell death

The results indicated that dihydromyricetin decreased the number of HL-60 cells in a dose-dependent manner. Figure 8 shows the effect of dihydromyricetin induced apoptosis in HL-60 cells. The nuclear morphology of untreated and treated cells is shown in Figure 11A using acridine orange staining, respectively. Acridine orange staining showed apoptotic bodies when cells were treated with 200 μM dihydromyricetin for 48 h. Cells undergoing apoptosis revealed a characteristic cleavage of DNA into oligonucleosome fragments manifesting as DNA laddering, a hallmark of apoptosis. The addition of dihydromyricetin to HL-60 cells resulted in the appearance of internucleosomal DNA ladder (Fig. 11B).

Dihydromyricetin induces apoptosis via intrinsically mediated pathway

The effects of dihydromyricetin on the protein expression of Bax, caspase-9, caspase-3, and PARP in HL-60 cells are shown in Figure 12. Dihydromyricetin treatment at 100 μM for 48 h has no significantly decreased of Bax (pro-apoptotic protein) expression in comparison to the control. Moreover, the results showed that exposure of HL-60 cells to dihydromyricetin (100 μM, 6 h) caused the degradation of pro-caspase-3, which generated a fragment of caspase-3. Dihydromyricetin treatment at 100 μM for 6 h significantly increased the degradation of PARP in comparison to the control.

The cell cycle effect of dihydromyricetin **treated in HL-60 cells**

The addition of dihydromyricetin to HL-60 cells possibly has the effects on cell cycle. We examined the effect of dihydromyricetin in the regulation of cell cycle. After being treated with dihydromyricetin for the indicated time. G1 phase proteins were detected by immunoblotting with cyclin E and cyclin D antibodies, or the S and G2/M phase proteins with cyclin A and cyclin B antibodies. The results revealed that decreased the expression of cyclin D and E after the treatment of dihydromyricetin (Fig. 13). These findings were consistent with G1 arrest.

Effects of *Pinus morrisonicola* Hayata extract prevents ultraviolet (UV) radiation -induced zebrafish embryos death

In order to determine whether the *Pinus morrisonicola* Hayata extract has an effect on zebrafish embryos, we exposed zebrafish embryos to UV (9000 J/m^2) after treated *Pinus morrisonicola* Hayata extract (250 μg/ml, 500 μg/ml, and 1000 μg/ml) for 15 minutes (Fig. 14). The results indicated that *Pinus morrisonicola* Hayata extract has no cytotoxicity on zebrafish embryos, and *Pinus morrisonicola* Hayata extract can prevent UV induced embryo death in a dose-dependent manner

討論 (Discussion)

There are few reports regarding the biological effects of *Pinus morrisonicola* Hayata extract have been found so far. Results from this study indicated that *Pinus morrisonicola* Hayata extract, the partition of water phase and its compounds, pinocembrin, tiliroside, and chrysin, inhibiting cancer cell growth and promoting apoptosis. However, the literature regarding the effects of *Pinus morrisonicola* Hayata extract and its compounds on cell population growth and apoptosis in human cancer cells remains unclear. The effects of *Pinus morrisonicola* Hayata extract and its compounds on cell growth inhibition and apoptosis in human cancer cells were further investigated in this study. We found that *Pinus morrisonicola* Hayata extract caused a significant decrease in the rate of cell population growth of human cancer cells at 24 h (Fig. 1A, B). However, *Pinus morrisonicola* Hayata extract did not affect the survival of normal peripheral blood mononuclear cells (Fig. 1C). Pinus species are a rich source of phenolic compounds, such as tectochrysin, ampelopsin, pinocembrin, pinostrobin, strobopinin, pinomyricetin, tiliroside, taxillusin, chrysin, strobochrysin, and trifolin (Jeffrey et al., 1999). In this study, quantitative determination of phenolic compounds in *Pinus morrisonicola* Hayata extract by HPLC indicated that *Pinus morrisonicola* Hayata extract contained pinocembrin, tiliroside, and chrysin (Fig. 6). There are some literatures on the inhibition of cancer cell growth with phenolic compounds from pinus species (Nyman, 1985; Premalatha and Sachdanandam, 1999; Soleas et al., 2002). Therefore, we investigated the inhibition of cancer cell growth of *Pinus morrisonicola* Hayata extract and its phenolic compounds in HL-60 cells. The data indicated that *Pinus morrisonicola* Hayata extract, pinocembrin, tiliroside, and chrysin dramatically inhibited on the cell population growth of HL-60 cells (Fig. 1A and Fig. 7). The water extracts of *Pinus morrisonicola* Hayata containing certain anti-oxidant substances are capable of scavenging the superoxide radical, and furthermore, they show anti-tumor activity (Hsu et al., 2005). Our results also indicated the effect of *Pinus morrisonicola* Hayata extract partition of water phase induced apoptosis in leukemia cancer cells (Fig. 5). Apoptosis was detected in HL-60 cells treated with *Pinus morrisonicola* Hayata extract at indicated dose as evidenced by the detection of nuclear fragmentation, DNA laddering and PI staining analyzed by flow cytometry (Fig. 3). *Pinus morrisonicola* Hayata extract induced apoptosis is mainly mediated by the degradation of pro-caspase-9 and pro-caspase-3, which generated a fragment of caspase-9 and caspase-3 (Fig. 4). In the similar results, chrysin-induced and dihydromyricetin-induced apoptosis are also mainly mediated by the degradation of pro-caspase-3, which generated a fragment of caspase-3 (Fig. 9, Fig. 12). Aside from

caspases mediated apoptosis, recent researches have shown that some anticancer drugs induce apoptosis in cancer cells through caspase-independent pathways. It has been found that during apoptosis, mitochondria membrane potential decreases and pro-apoptotic factors, including pro-caspases and caspase-independent factors (such as apoptosis-inducing factors, AIF), were released from mitochondria into cytosol (Huang et al., 2005; Kawagoe et al., 2002). The expression of critical cell cycle regulatory proteins cyclin D1 and cyclin E was significantly decreased and the G1 phase was blocked in HL-60 cells following *Pinus morrisonicola* Hayata extract treatment (Fig. 2). In the similar results, chrysin-induced and dihydromyricetin-induced cell arrests are also mainly mediated by the cell cycle regulatory proteins cyclin D1 and cyclin E (Fig. 10, Fig. 13). It is the first report, describing the mechanism of *Pinus morrisonicola* Hayata extract mediated inhibition of leukemia cancer cell growth. In this study, we have shown that *Pinus morrisonicola* Hayata extract exerts a significant effect on inhibition of cell growth and induction of apoptosis in leukemia cancer cells mediated by cell cycle and apoptosis regulatory proteins. Future in vivo study will reveal the anticancer efficacy of *Pinus morrisonicola* Hayata extract in leukemia cancer animal models. In addition, our data showed that *Pinus morrisonicola* Hayata extract at 500 μg/ml did not have significant cytotoxicity on peripheral blood mononuclear cells and zebrafish embryos (Fig. 1C and Fig. 14). However, at this concentration, it significantly inhibited the cell growth of human leukemia cells (Fig. 1A, B). These data suggest that *Pinus morrisonicola* Hayata extract, as natural substances with powerful growth inhibition and induce-apoptosis effects on leukemia cells, will be a good candidate for chemoprevention or chemotherapeutic adjuvant in the future. In summary, we have shown that *Pinus morrisonicola* Hayata extract exerts a significant effect on inhibition of cell growth and induction of apoptosis in leukemia cancer cells mediated by cell cycle and apoptosis regulatory proteins. The purification, isolation and mechanism of these active components in *Pinus morrisonicola* Hayata are of interest for further investigation, which will be finding out in our future researches.

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黃雯雯，2002，松針及蜂膠抽提物抑制人類血癌細胞株生長之研究，中國醫藥大學中國藥學研究所博士論文。

圖表（Figures）

Figure 1. Effects of *Pinus morrisonicola* Hayata extract on the cell viability of HL-60 cells (A), JK cells (B), and PBMCs (C). Cells were treated with 5-500 μg/ml *Pinus morrisonicola* Hayata extract for 24 h.

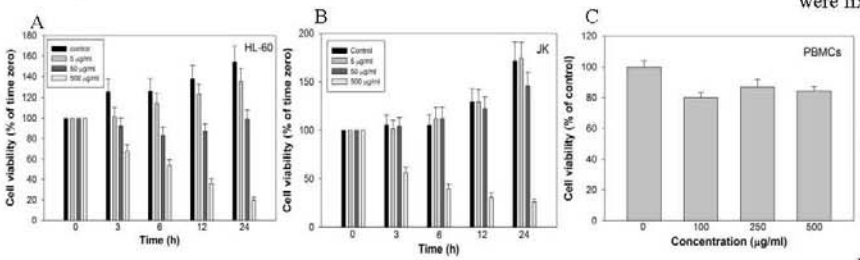


Figure 2. *Pinus morrisonicola* Hayata extract induced cell arrest in HL-60 cells. (A) Effects of *Pinus morrisonicola* Hayata extract on the protein expression of cyclin A, B1, D1, E, CDK1, 2, 4 (B) Cells were fixed, stained with PI and analyzed by flow cytometry.

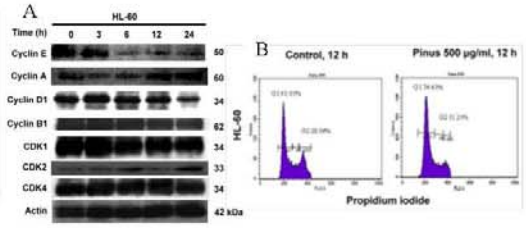


Figure 3. *Pinus morrisonicola* Hayata extract induced apoptosis in HL-60 cells. (A) Stained with acridine orange and then detected by fluorescence-microscope. (B) DNA fragmentation was analyzed by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining. M, DNA ladder marker. (C) Cells were fixed, stained with PI and analyzed by flow cytometry.

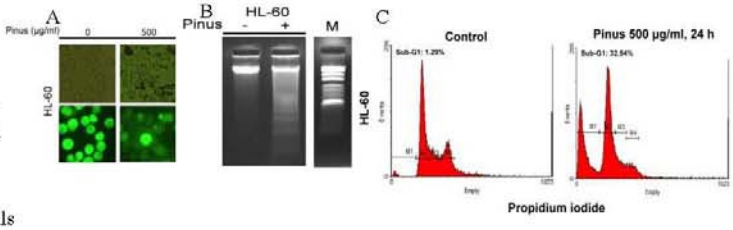


Figure 4. Effects of *Pinus morrisonicola* Hayata extract on the protein expression of Bax, Bcl-2, caspase-9, caspase-3, and PARP in HL-60 cells. Cells were treated with 500 μg/ml *Pinus morrisonicola* Hayata extract for 0.5-18 h.

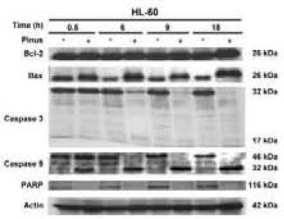


Figure 5. *Pinus morrisonicola* Hayata extract partition of water phase induced apoptosis in HL-60 cells. (A) Effect of *Pinus morrisonicola* Hayata extract partition of water phase on the cell viability of HL-60 cells. (B) Stained with acridine orange and then detected by fluorescence-microscope. (C) DNA fragmentation was analyzed by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining. M, DNA ladder marker.

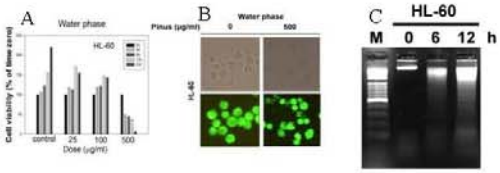


Figure 6. Typical high-performance liquid chromatography (HPLC) profile of (A) *Pinus morrisonicola* Hayata extract and (B) other pine extract standard. Peaks 1, 2 and 3 are pinocembrin, tiliroside, and chrysin, respectively. Ultraviolet-visible spectrophotometric array detector set at 290 nm. (C) The MS-MS chromatograms of the pure pine extracts are reported in figure 6C.

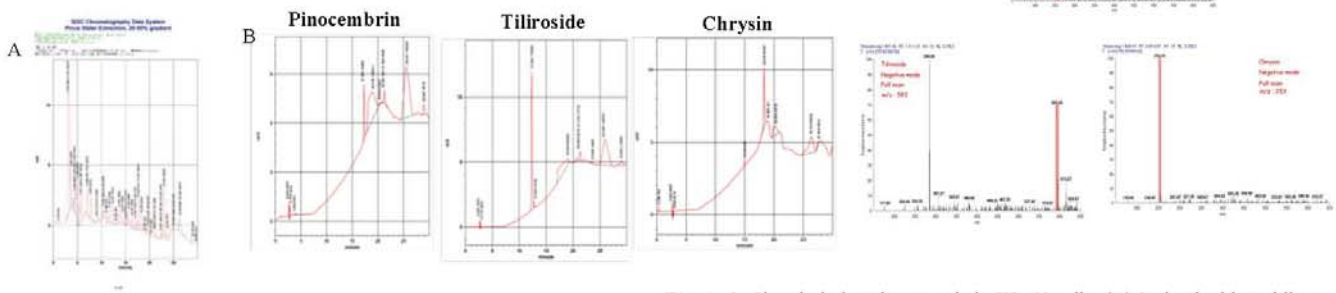


Figure 7. Effects of dihydromyricetin, pinocembrin, tiliroside, and chrysin on the viability of HL-60 cells. Cells were treated with 0-200 μM dihydromyricetin, pinocembrin, tiliroside, or chrysin for 48 h.

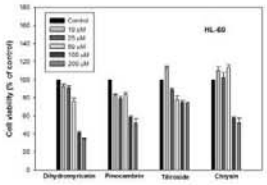


Figure 8. Chrysin induced apoptosis in HL-60 cells. (A) Stained with acridine orange and then detected by fluorescence-microscope. (B) DNA fragmentation was analyzed by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining. M, DNA ladder marker.

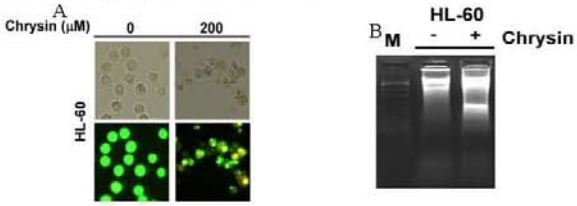


Figure 9. Effects of chrysin on the protein expression of caspase-9, caspase-3, and PARP in HL-60 cells. Cells were treated with 100 μM chrysin for 0.5-24 h.

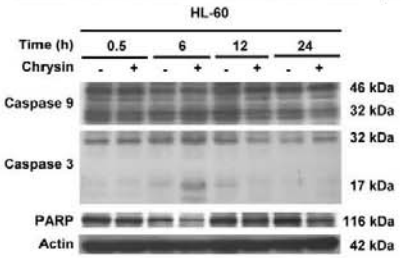


Figure 10. Effects of chrysin on the protein expression of cyclin A, B1, D1, E. Cells were treated with 100 μM chrysin for 0.5-24 h.

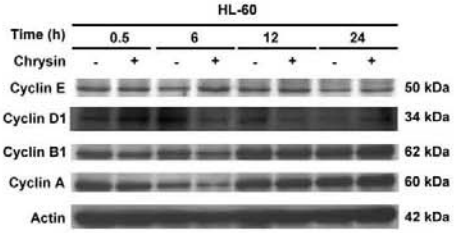


Figure 11. Dihydromyricetin induced apoptosis in HL-60 cells. (A) Stained with acridine orange and then detected by fluorescence-microscope. (B) DNA fragmentation was analyzed by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining. M, DNA ladder marker.

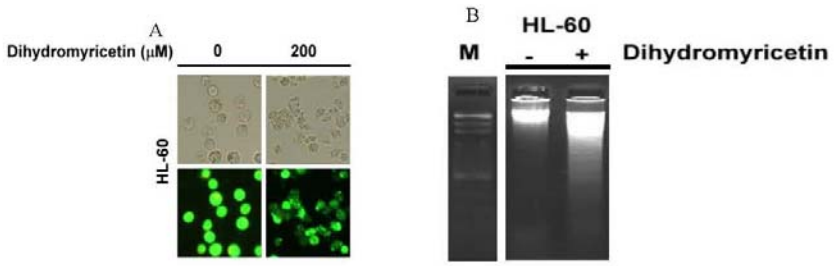


Figure 12. Effects of dihydromyricetin on the protein expression of Bax, caspase-9, caspase-3, and PARP in HL-60 cells. Cells were treated with 100 μM dihydromyricetin for 0.5-24 h.

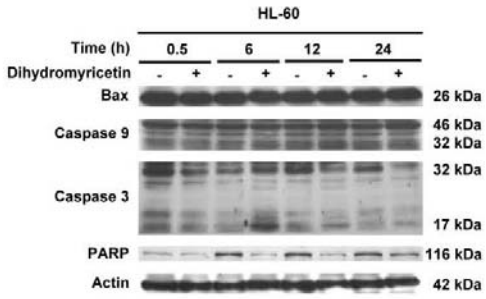


Figure 13. Effects of dihydromyricetin on the protein expression of cyclin A, B1, D1, E. Cells were treated with 100 μM dihydromyricetin for 0.5-24 h.

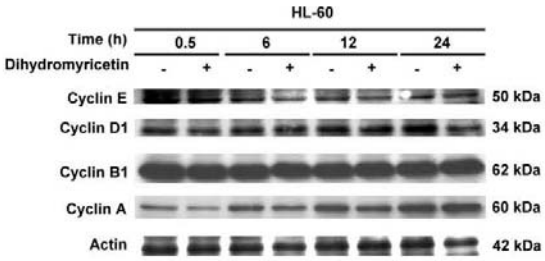
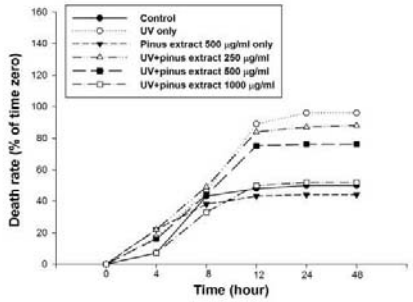


Figure 14. Effects of *Pinus morrisonicola* Hayata extract prevent ultraviolet radiation -induced death of zebrafish embryos.



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：劉光耀	計畫編號：98-2324-B-040-001-
計畫名稱：台灣本土五葉松萃取物免疫功能評估及促進其商品產業化	

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	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%		
	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	0	0	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	2	2	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%		

其他成果 (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。)	無

	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)