

行政院國家科學委員會專題研究計畫 成果報告

多花滇南蛇藤之抗癌成分研究

計畫類別：個別型計畫

計畫編號：NSC94-2314-B-039-033-

執行期間：94年12月01日至95年07月31日

執行單位：中國醫藥大學生物科技學系

計畫主持人：翁靖如

共同主持人：翁舷誌

報告類型：精簡報告

處理方式：本計畫可公開查詢

中 華 民 國 95 年 10 月 27 日

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

(計畫名稱)

多花滇南蛇藤之抗癌成分研究

計畫類別： 個別型計畫  整合型計畫

計畫編號：NSC 94-2314 - B - 039 - 033 -

執行期間： 94 年 12 月 1 日至 95 年 7 月 31 日

計畫主持人：翁靖如

共同主持人：翁舷誌

計畫參與人員：翁靖如、翁舷誌

成果報告類型(依經費核定清單規定繳交)： 精簡報告  完整報告

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執行單位：中國醫藥大學生物科技學系

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## 一、研究計畫中英文摘要：

### (一) 計畫中文摘要。

Keywords: *Celastrus paniculatus*, anti-cancer

#### 中文摘要:

癌症中，細胞增殖及設定性凋亡的平衡干擾而使得本身具有基因異常的細胞沒有死亡，而存活下來。很多毒殺性及荷爾蒙方面的治療，如放射線，特異性的毒殺癌細胞藉由設定性凋亡來造成不可逆的細胞損害，因此尋找與設定性凋亡相關的抗癌藥物是非常重要的。

在此計劃中已針對衛矛科植物中的多花滇南蛇藤(*Celastrus paniculatus*)進行活性成分分離及鑑定，並進行抗癌活性之探討。由於 *Celastrus* 此屬中在文獻搜索中發現具有殺蟲，抗發炎成分，抗氧化之配醣體外，還有 sesquiterpene pyridine alkaloid 之抗癌成分。在對 *C. paniculatus* 甲醇初萃物進行初步藥理活性篩選，發現對於一系列的 cancer cell lines 呈現有意義的抑制活性。目前藉由矽膠色層分析方式，已分離得到固醇類與萜類化合物，正進行結構分析鑑定與藥理活性試驗中，以期發展為臨床用藥。

### (二) 計畫英文摘要。

#### 英文摘要:

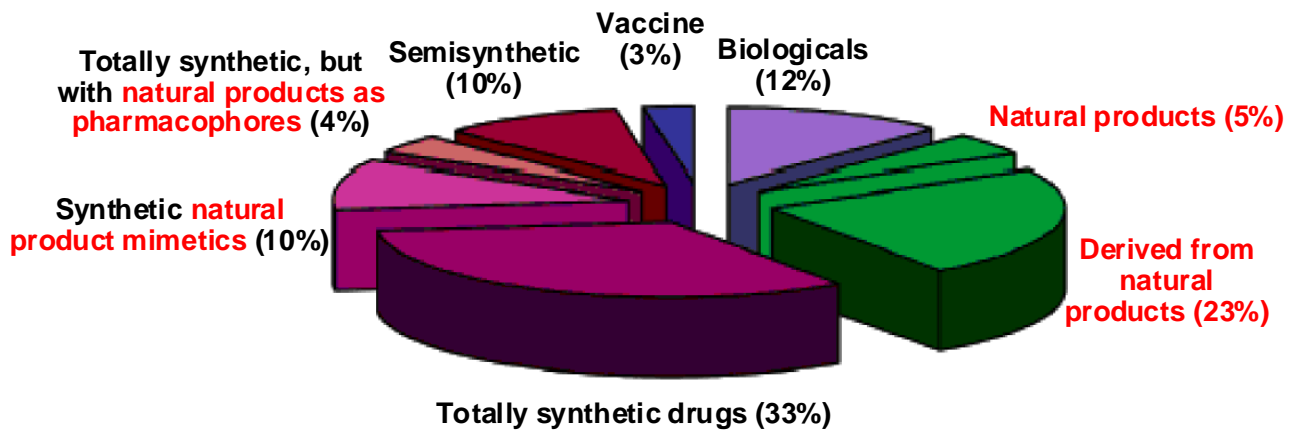
In cancer, the balance between proliferation and programmed cell death is disturbed, and defects in apoptotic pathways allow cells with genetic abnormalities to survive. Most cytotoxic and hormonal treatment, as well as radiation, ultimately kill cancer cells by causing irreparable cellular damage that triggers apoptosis.

The methanolic extracts of the plant of *C. paniculatus* showed significant anti-cancer activity by using in vitro testing. The family *Celastraceae* is well known to have various constituents, some of which exhibit insecticidal, anti-inflammatory, antioxidant, and antitumor activity. The results of the screening data have been shown that methanolic extracts of *C. paniculatus* possess some anti-cancer potential and worth to be investigated.

In this proposal, we have already isolated and characterized some steroids and terpenoids from *C. paniculatus* by using chromatographic method. Meanwhile, the identification and characterization of these structures and the pharmacological study are undertaken to establish the mechanisms of action of these compounds and try to develop a drug for clinical use.

## 二、報告內容：

隨著工商業的發達，人們的健康不再是以壽命的延長為基準，而是著重於身體老化或疾病發生之處理或預防。而最近幾年，癌症於國人之十大死因中持續佔排名第一的事實，促使許多學者專家投注心力，希望藉由分子生物或藥品研發的方式得以達成減緩癌症之發生率或增加其緩解程度。而由著名期刊 *J. Nat. Prod.* 所發表之資料中，顯示近二十年所研發之新藥多以天然物為主體，進而衍生(1)(圖一)。由此推測中草藥所蘊藏豐富的資源裡，因含有各類的成分與化合物，廣泛之藥理活性受到高度重視。



因此對於天然植物之探索中，已於 *Artocarpus heterophyllus* 及 *Artocarpus communis* 之抽出物分別分離得到十二個及十個黃酮類成分。其中以 artocarpanone 對於 NO 生成及 iNOS 蛋白質於 RAW 264.7 cells 之表現有明顯抑制，由此推測 artocarpanone 對於以 lipopolysaccharide 誘發巨噬細胞之 NO 形成，可能是經由抑制 iNOS 蛋白質的表現 (2)。且將黃酮類成分作人體之抗小板凝集試驗上，發現 dihydroartemoxanthone，artochamins B 與 artocommunol CC 對於 adrenaline 所誘發的凝集有明顯的抑制(3)。

此外，利用天然成分進行半修飾的合成方面，已合成一系列 Indole-3-carbinol 衍生物。這十三個新衍生物已透過藥理試驗得到的資料，顯示部份化合物對於 prostate cancer cell line (PC-3) 有很強的抑制活性，IC<sub>50</sub> 值分別為 6.4 及 8.9 μM。由此 data 已證實 Indole-3-carbinol derivatives 比 parent compound 的活性好，且有將近 10 倍的抑制活性之改善，目前已將成果及藥理機制探討預備投稿中。

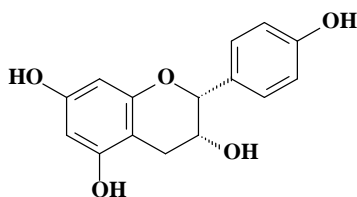
而在藥物與分子標靶的課題上，也探討了 α-tocopheryl succinate 在前列腺癌細胞中，部分經由 Bcl-xL/Bcl-2 功能抑制而誘發設定性細胞凋亡的發生。利用對 α-tocopheryl succinate 的化學部份修飾後的結構進行體外活性試驗及分子模擬分析，發現 α-tocopheryl succinate 對前列腺癌細胞的設定性細胞死亡與 bcl-xl/bcl-2 的功能被抑制有關，並且已將此資料投稿於 *J. Biol. Chem.* (4)。

而 PPAR<sub>γ</sub> agonists 的研究課題中，針對許多文獻提出部份的 PPAR<sub>γ</sub> agonist 具有抗腫瘤活性，但詳細機制不明。於是藉由合成一系列 PPAR<sub>γ</sub>-inactive TZDs 類似物在不同癌細胞株測試後，發現修飾後之化合物與原始物相較下，仍保有其誘發設定性細胞凋亡活性，卻沒有 PPAR<sub>γ</sub> 活性，且這些 TZDs 還會影響 cyclin D1 及 caspase-8-related FLICE-inhibitory protein (FLIP) 裂解(5)。

由於乳癌的治療，與 HER2 過度表現有關，雖現今已知人類的單株抗體 trastuzumab

是會標靶於 tyrosine kinase receptor HER2 的細胞外 domain，並已被成功的應用於 HER2 過度表現之擴散性乳癌治療，但仍需克服 trastuzumab 產生抗性的機會。因此藉著 celecoxib 所衍生的新化合物應用在 HER2 表現之細胞株，發現具有加強 trastuzumab 之療效，而提供一個對 trastuzumab 產生抗性之乳癌之用藥策略(6)。

在文獻搜尋中，發現衛矛科的 *Celastrus* 屬含有 sesquiterpenoids, alkaloid 及 flavan-3-ol glycoside (7-12)。部份成分中的(-)-epiafzelechin (1)具有抑制 COX-1 的活性及 NO 的生成，其 IC<sub>50</sub> 值分別為 0.27 及 0.23 μM，相當強(13, 14)。COX-1 與 COX-2 均屬於炎症反應之重要參與酵素，且發炎反應中的 macrophage 也在癌症的發生扮演了重要角色。例如:活化的 macrophage 會產生 TNF 及 Nitric oxide，而這兩種的化學傳遞物質都會引起 tumor cell killing (15, 16)。而且 Nitric oxide 還會影響離子通道的活性進而影響粒線體膜電位之平衡，由此可知炎症與癌症的緊密相關性(16)。若能在 macrophage 內能抑制 NO 生成，即可能發展成抗炎及 cancer chemopreventive agent。



1

此株植物在先前文獻中並沒有全草的成分報告，而偏重於種子成分分離，此外也沒有細胞毒成分報告，且依化學篩選成分分布相當多，值得進行活性成分之分離。綜合上述資料，故進行 *Celastrus paniculatus* 之活性成分研究，尋求活性物質，以提供臨床使用開發新的醫療藥物。

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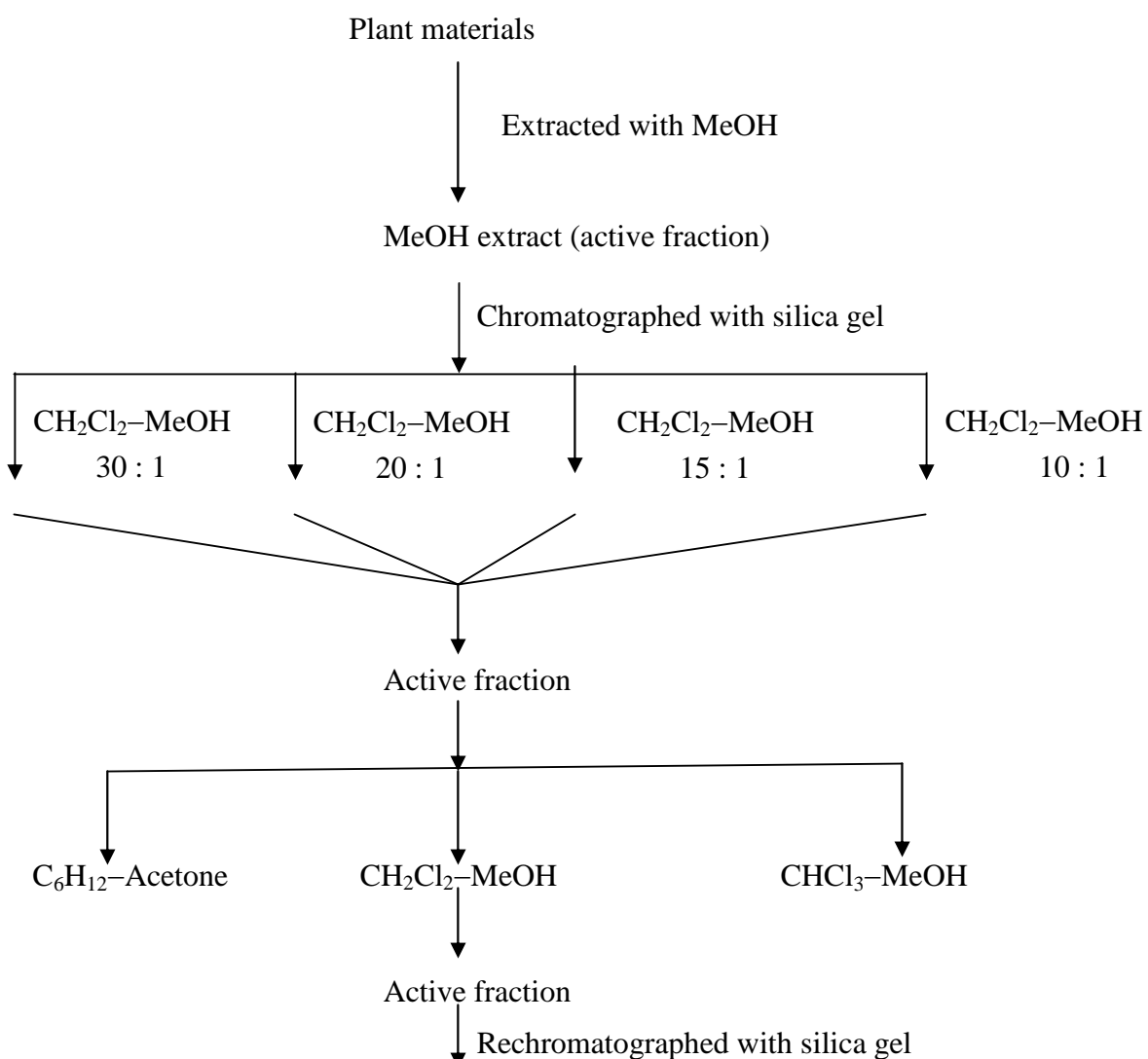
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## 研究方法

### Isolation and structure determination

Plant material will be extracted with appropriate solvent. The solvent extract will be evaporated in vacuo, yielding a residue, which will be separated by Si gel column chromatography to give various compounds. The  $\text{CHCl}_3$ , EtOAc, and *n*-BuOH fractions will be separated and purified on Si gel or reversed-phase gel, to give various compounds (Scheme 1). Various pure compounds will be characterized with UV, MS, IR, 1D-NMR.



Scheme 1

## Antitumor activity

The present studies will be designed to investigate the anti-tumor activities of natural products and compounds. In this proposal, the following approaches will be taken:

- (1) The direct cytotoxic activity of those compounds in human hepatoma, Hep 3B, human lung carcinoma, A549, human breast carcinoma, MCF-7, and cervical carcinoma, HT-29 cells.
- (2) Flow cytometry analysis.
- (3) The cytotoxic mechanisms of those compounds in the above human cancer cell lines *in vitro*.

## Tumor cell growth inhibition assays

The cytotoxicity was determined by colorimetric MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-1H-tetrazolium bromide) assay as described previously (1, 2). Briefly, cells ( $5 \times 10^3$ /well) were plated in the 96-well plates and incubated in medium for 6 h. Fifty microliters of serial tested drug dilutions was added. The cells were incubated for 5 or 6 days at 37°C and then pulsed with 10  $\mu$ L of MTT (5 mg/mL; Sigma, St. Louis, MO) and incubated for an additional 4 h at 37 °C. Reduced MTT was measured spectrophotometrically by a Dynatech MR5000 microplate reader (Dynatech Laboratories, VA) at 550 nm after lysis of cells with 100  $\mu$ L of 10 % SDS in 0.01M HCl. Control wells contained medium plus cells (total absorbance) or medium alone (background absorbance) cell death was calculated as the percentage of MTT inhibition:

Cell death was calculated as the percentage of MTT inhibition:

$$\% \text{ inhibition} = \frac{\text{mean control absorbance}}{1 - \text{mean experimental absorbance}} \times 100$$

## Western blot analysis of PARP cleavage

Drug-treated cells were collected, washed with ice-cold PBS, and resuspended in lysis buffer [20 mM Tris-HCl (PH 8), 137 mM NaCl, 1 mM CaCl<sub>2</sub>, 10 % glycerol, 1 % Nonidet P-40, 0.5 % deoxycholate, 0.1 % SDS, 100  $\mu$ M 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin at 10  $\mu$ g/mL, and aprotinin at 10  $\mu$ g/mL]. Soluble cell lysates were collected after centrifugation at 1500g for 5 minutes. Equivalent amounts of protein (60-100  $\mu$ g) from each lysate were resolved in 10 % SDS-polyacrylamide gels. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-PARP antibodies, as described above (3).

## Apoptosis detection by an enzyme-linked immunosorbent assay (ELISA)

Induction of apoptosis was assessed by using a Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany) by following the manufacture's instructions. This test is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death. In brief,  $2.5 \times 10^6$  cells were cultured in a T-75 flask 24 hours before the experiment. Cells were washed twice in 5 mL of serum-free RPMI-1640 medium and then treated with a test agent or the DMSO vehicle, as indicated. Both floating and adherent cells were collected, and cell lysates equivalent to 10<sup>4</sup> cells

were used in the ELISA (3).

### Flow cytometry

For cell apoptosis analysis, cells ( $2 \times 10^6$ ) were seeded into six-well trays. The cells were treated with tested drug for 48 h and harvested. The cell pellets were resuspended in Hepes-buffer saline (HBS, 160 mM NaCl, 2.7 mM KCl, 6 mM glucose, 10 mM Hepes, 0.1% BSA, pH 7.3) and stained with dye Merocyanine 540 (MC540; Acros, Belgium) in the dark for 10 min. MC540, similar to Annexin V, can bind phosphatidylserine of extroverted inner membrane component fluorescence-activated cell sorter (FACScan; Becton-Dickinson immunocytometry system, CA).

### DNA fragmentation assay

After tested drug ( $8 \mu\text{g/mL}$ ) treatment for 48 h, cell in 150-mm plates were harvested and washed with PBS. After addition of  $100 \mu\text{L}$  lysis buffer [1% of NP-40 (Sigma) in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5] and mixing, the cell lysates were centrifuged at 14,000 rpm for 5 min and the supernatants were collected. The supernatants were incubated with  $50 \mu\text{L}$  of RNase A (20 mg/mL) and  $20 \mu\text{L}$  of SDS (10%) at  $56^\circ\text{C}$  for 2 h. Then,  $35 \mu\text{L}$  of proteinase K (20 mg/mL) was added and incubated at  $37^\circ\text{C}$  overnight. DNA fragments were precipitate after addition of  $150 \mu\text{L}$  of 10 M  $\text{NH}_4\text{OAc}$  and 1.2 mL of 100% ethanol at  $-20^\circ\text{C}$  overnight. After centrifuging and drying, the DNA pellets were resuspended in  $15 \mu\text{L}$  Tris-EDTA buffer and electrophoresed on a 1% agarose gel in TBE buffer at 30 V for 8 h, DNA ladder was observed after staining with ethidium bromide solution and exposing to the UV light (3).

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### 結果與討論

在針對衛矛科 *Celastrus paniculatus* 之粗抽萃取物進行細胞毒篩選活性試驗，發現其全草的 MeOH 初抽物對各種癌細胞具有明顯的毒殺作用，尤其是對 Hep 3B 的毒性較強 (Table 1, 2)，故進行田野採集此植物。而在進行 *C. paniculatus* 之甲醇抽出物以矽膠進行多次之色層分析，除發現富含植物之常見成分如  $\beta$ -sitosterol 與 lupeol 外，還發現三種固醇類與四種 terpene 類化合物，並進行結構分析與鑑定中。並且於成分鑑定後，會繼續做藥理活性試驗，以期獲得結構與活性之相關性，以利藥品開發。



**Table 1.** MeOH crude extract of *Celastrus paniculatus* 對各種癌細胞之生長抑制百分比.

Crude ext. ( $\mu\text{g/ml}$ )	Cell line			
	MCF-7	A549	Hep 3B	HT-29
100	96	97	-	94
50	70	67	91	75
25	59	36	75	62
12.5	50	-	71	47
6.25	33	-	62	37
IC <sub>50</sub>	19.4	37.3	5.25	17.1

**Table 2.** CHCl<sub>3</sub> crude extract of *Celastrus paniculatus* 對各種癌細胞之生長抑制百分比.

Crude ext. ( $\mu\text{g/ml}$ )	Cell line			
	A549	MCF-7	Hep 3B	HT-29
200	93.3	-	-	93
100	69.7	74	87	66
50	53	63	68	46
25	41	40	36	40
12.5	35	-	-	-
IC <sub>50</sub>	50.6	36.5	36.5	57.3

#### 計畫成果自評

本計畫對多花滇南蛇藤之抗癌成分的研究，其研究內容與原計畫十分相符，乃利用蘊藏於天然資源的具藥物開發潛力之化合物，以純化與分析方式獲得純化合物，並將純化合物與癌細胞株之活性試驗篩選結合，以期獲得具抗癌之活性成分。

而在預期目標的執行上，不僅由此株植物的粗萃取物的藥理試驗已獲得其具有抗癌的活性成分之資料外，也在計畫參與的人員包括教授，助理教授及大學部學生的共同努力下，已得到固醇類與 terpene 之多種成分並進行結構之鑑定與分析。而對大學部學生而言，則是使他們有機會得以增強分離技術上及化學結構鑑定的能力與經驗。未來，這些研發成果不只能發表於學術期刊外，也能有助於其他新藥開發的應用設計。