行政院國家科學委員會補助專題研究計畫成果報告

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中文摘要

銳葉楊梅葉之 70%丙酮萃取物對子宮頸癌細胞具有抑制作用且會誘導其凋亡。然而在相同濃度時對正常的子宮頸纖維母細胞毒性較小，且高濃度時其作用機制亦為誘導細胞凋亡。繼而利用抗癌活性追蹤的方式，經過一連串的層析管柱分離純化之步驟，第一次由銳葉楊梅葉中分離出(-)-epigallocatechin 3-O-gallate (EGCG) 及 prodelphinidin A-2, 3'-O-gallate (PAG)，其產率分別為 0.0036%與 0.0013%。這兩個成分在濃度 5~40 ìg/ml 下對子宮頸癌細胞呈現濃度依存性的關係，而其在 48 小時抑制百分之五十細胞生長所需濃度分別為 9.34 ìg/ml 及 38.50 ìg/ml，對於子宮頸癌細胞的選擇指數(2.16, 0.99)則是 EGCG 高於 PAG。進一步探討其作用機制發現： EGCG 在 24 小時濃度為 10 ìg/ml 即會引起 DNA 斷鏈且有染色質濃縮的現象；而 PAG 濃度為 40 ìg/ml 時亦會造成凋亡小體的產生，由此推測 EGCG 與 PAG 的作用機制皆為誘導細胞凋亡。而以 caspase-3-specific inhibitor 預處理細胞之後，結果能有效抑制 PARP 之 cleaved fragment(85kDa)產生，證實 EGCG 與 PAG 的細胞凋亡機制為 caspase-3 dependent pathway。另外，以 catalase (375U/ml) 培養細胞後，發現能有效降低 EGCG 所造成的細胞毒性，而 PAG 則無此現象。因而推論 EGCG 可能是經由本身產生的自由基進而誘導子宮頸癌細胞進行凋亡，而其作用機制與 caspase-3 路徑有關。

Abstract

Myrica rubra var. *acuminata* is a native shrub widely distributed and is used as folk medicine in Taiwan for stomach disorders and diarrhea. Column chromatography combined with cytotoxic bioassay-guided fractionation was performed to isolate the antitumor principles from fresh leaves of *M. rubra* var. *acuminata.* The 20% MeOH eluate fraction (D-20) of *M. rubra* var. *acuminata.* inhibited the viability of HeLa and P-388 cells in an *in vitro* assay and an *in vivo* P-388 tumor-bearing CDF_1 mice model. The percent increase in life span (% ILS) of D-20 was greater than 125%. (-)-Epigallocatechin 3-*O*-gallate (EGCG) and prodelphinidin A-2, 3'-O-gallate (PAG) were isolated from D-20 as the antitumor principle components. Both compounds can inhibit the growth of HeLa cells, but EGCG had lower cytotoxic effects in normal cervical fibroblasts than did PAG. Moreover, pretreatment with a caspase-3-specific inhibitor prevented EGCG- and PAG-induced PARP cleavage. In view of these results, we suggest that EGCG and PAG can induce apoptosis in HeLa cells, and that activation of caspase-3 may provide a mechanistic explanation for their cytotoxic effects. Therefore, we suggest that D-20 fraction extract is good for health, and that *M. rubra* var. *acuminata* is an economically valuable plant.

Keywords: *Myrica rubra* var. *acuminata*; Myricaceae; apoptosis; HeLa cells; P-388 cells; (-)-epigallocatechin 3-O-gallate; prodelphinidin A-2, 3'-O-gallate

緣由與目的

Myrica rubra (Myricaceae) is widely distributed in Taiwan, and there is one variety, *M. rubra* $S_{IEB.}$ et Z_{UCC} var. *acuminata* N_{AKAI}(*1*). The bark of *M. rubra* has been used locally as an astringent, an antidote, and an antidiarrheic in Chinese traditional medicine (*2*). Previously, several flavonoids, tannins, triterpenes, and diarylheptanoids were isolated from the bark of *M. rubra* (*3-8*). Pharmacological studies of this medicinal plant have reported that its methanolic extract showed protective effects on CCl_4- and α-naphthylisothiocyanate-induced liver injury, whereas the 50% aqueous enthanolic extract and some constituents inhibited melanin biosynthesis and showed anti-androgenic activity (*9*). However, the constituents of *M. rubra* var. *acuminata* have not been investigated.

In our previous study, we screened the cytotoxicity effects of 70% acetone extracts of medicinal plants on HeLa cells using an MTT assay (*10*). The 70% acetone extract of *M. rubra* var. *acuminata* showed greater cytotoxic

effects on cervical tumor cells than on normal cells. In the present study, cytotoxicity bioassay-guided fractionation was performed to isolate the antitumor principles from *M. rubra* var. *acuminata*, and their mechanism for inducing the cell death mode in tumor cells was investigated.

Several chemicals can induce tumor cell death; however apoptosis is an efficient strategy for cancer chemotherapy. The apoptotic mode involves the active participation of affected cells in a self-destruction cascade that culminates in DNA degradation *via* endonuclease activation, nuclear disintegration, and formation of "apoptotic bodies" that involve the cell remnants. These apoptotic bodies are rapidly cleaned from local tissues by macrophages (*11*). However, little is known about the regulation and induction of apoptosis by natural products. In the present study, the human cervical carcinoma cell line (HeLa) and mouse leukemia cell line (P-388), were differentially susceptible to apoptosis induced by a natural product. Cell death was detected and identified using an MTT assay, propidium iodide staining followed by flow cytometry, DNA electrophoresis, and poly(ADP-ribose) polymerase (PARP) proteolysis by Western blot assay (*12*).

While many compounds have been shown to inhibit the proliferation of mammalian cells in culture, only a small proportion of these demonstrate significant selectivity *in vivo* even in the most chemosensitive animal tumor models. Therefore, the antitumor effects of the cytotoxic components will be evaluated using a P-388 tumor-bearing CDF_1 mice model (*13*). In summary, we have isolated natural products from *M. rubra* var. *acuminata* which can induce apoptosis in tumor cells and prolong the survival time of P-388-bearing mice.

結果與討論

The 70% aqueous acetone extract of *M. rubra* var. *acuminata* leaves was strongly cytotoxic to HeLa cells, with on IC_{50} of 21.69 μg/ml for 48 h. Based on the bioassay guide for fractionation, the aqueous extract was chromatographed on a Diaion HP-20 column to give five eluted fractions, and their cytotoxic effects. Of the five fractions, fraction II (20% MeOH-eluted fraction, D-20) displayed the strongest cytotoxic effect. Therefore, the antitumor effects of D-20 were evaluated using murine P-388 leukemia in *in vitro* and *in vivo* models. In an *in vitro* assay, D-20 induced cell death and DNA fragmentation in a dose-dependent manner in P-388 cells with an IC_{50} of 24.8 μg/ml for 24 h. The effect of D-20 was evaluated for its *in vivo* antitumor activity against intraperitoneally implanted P-388 leukemia in CDF_1 mice. D-20 at a dose of 18.75 mg/kg b.w. prolonged the life span of P-388 tumor bearing CDF_1 mice by more 125% compared to normal saline-treated blank mice (Table 1). However, the high doses of D-20 caused a toxic reaction in these mice, D-20-treated group, such that the ILS% was below 100% (Table 1). In the 18.75 mg/kg D-20-treated group, the mean body weight of the mice was significantly lower than that of the blank group on days 9 to 18. According to the above results, D-20 can inhibit the growth of P-388 cells *in vitro* and *in vivo* and is the major antitumor fraction extract of *M. rubra* var. *acuminata*. Therefore, D-20 was rechromatographed on a Toyopearl HW-40(C) column, and the cytotoxic effects of each developing fraction. The higher yield and greater cytotoxic effect of the 60% MeOH-developed fraction was separated and purified using an ODS column to give (-)-epigallocatechin gallate (EGCG) and prodelphinidin A-2, 3'-O-gallate (PAG) (Fig. 1).

Table 1. Percentage increase in life span (%ILS) of D-20 treated P-388-bearing CDF_1 mice

Group	Survival time (day)	%ILS
Blank (normal saline)	24.3	100.0
D-20 (mg/kg)		
18.75	>> 60.0	>> 125.0
37.50	22.5	92.8
75.00	17.5	72.2

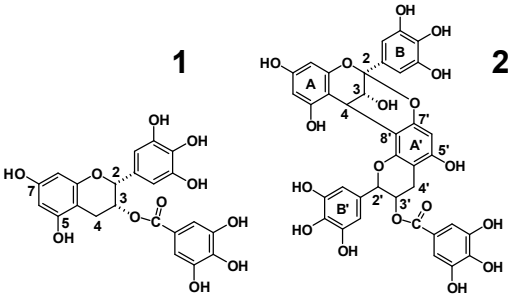

Fig. 2 Structures of (-)-epigallocatechin 3-*O*-gallate, EGCG (1) and prodelphinidin A-2, 3'-O-gallate, PAG (2).

EGCG and PAG were isolated from leaves of *M. rubra* var. *acuminata* as antitumor principles for the first time, and yields were 0.0036% and 0.0013%, respectively. The cytotoxic effects of these compounds exhibited dose-dependent effects at 5~40 ì g/ml in HeLa cells for 24, 48, and 72 h (Fig. 2). EGCG has fewer cytotoxic effects on primary normal cervical fibroblasts than on HeLa cells, but that was not the case for PAG (Table 2). Furthermore, the cytotoxic mechanisms of EGCG and PAG were measured by fluorescence flow cytometry. DNA fragmentation is a characteristic feature of apoptosis (*16*). Fig. 6 shows that EGCG and PAG induced DNA fragmentation at 10 to 80 ì g/ml in HeLa cells for 48 h.

Table 2. IC_{50} values of EGCG and PAG on HeLa and primary culture human normal cervical fibroblasts (NCF) after 48 h of treatment

	IC_{50} (μg/ml)		
Compound	HeLa	NCF	SI[1]
EGCG	9.34	20.18	2.16
PAG	38.50	38.77	0.99
Adriamycin	0.15	<0.15	<1.00

[1]SI: selectivity index, IC_{50} for NCF/ IC_{50} for HeLa. Adriamycin is a positive control drug.

Apoptosis produced the typical pattern of apoptotic poly(ADP-ribose) polymerase (PARP) cleavage: a catalytically active band of intact PARP at 116 kDa, and an active band at 85 kDa corresponding to the apoptotic cleavage product of PARP. PARP is proteolytically cleaved during apoptosis by caspase-3 (*17*) which reduces PARP's enzymatic activity (*18*), thereby inhibiting DNA repair. Treatment of HeLa cells with EGCG and PAG stimulated proteolytic cleavage of PARP in a dose-dependent manner. Pretreatment with 100 μM of a caspase-3-specific inhibitor (Ac-Asp-Glu-Val-Asp-aldehyde) for 2 h inhibited EGCG- and PAG-induced PARP cleavage. The above results suggest that EGCG and PAG can induce apoptosis in HeLa cells, and that activation of caspase-3 may provide a mechanistic explanation for their cytotoxicity effects.

EGCG is well known as a chemopreventive agent which is abundant in tea (*19*). In the present study, we first report that leaves of *M. rubra* var. *acuminata* contain EGCG, and that the D-20 extract can inhibit the growth of P-388 cells *in vitro* and *in vivo*. As previously reported (*9*) the 50% EtOH extract of leaves of *M. rubra* inhibited melanin biosynthesis *in vitro* and can possibly be used as a whitening agent for the skin. Therefore, we suggest that D-20 fraction extract is good for health, and that *M. rubra* var. *acuminata* is an economically valuable plant. In the future, EGCG will be used as a biosubstance to control the quality of the D-20 fraction extract.

成果自評

本論文結果：1. 活性篩選結果已發表於國內優良期刊 Nutr. Sci. J, 27(2), 109-117。 2. 活性成分分離結果正投稿於 J. Agar. Food. Chem。 3. 參加研討會論文，結果發表於 2002 年美國生藥學會。

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