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Antileukemic Activity of Selected Natural Products in Taiwan

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Abstract: The aim of the present study was to evaluate the antileukemic activity of six chemical classes of pure compounds present in commonly used medicinal plants in Taiwan — such as the genus *Plantago*. Studies were conducted on a series of human leukemia and lymphoma cell lines. Results showed that water soluble compounds (aucubin, caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid and vanillic acid) exhibited a weak antileukemic activity (IC_{50}: 26–56 μg/ml, SI: 2–11). On the other hand , water insoluble compounds such as triterpenoids (oleanolic acid and ursolic acid), monoterpene (linalool) and flavonoid (luteolin) possessed strong activity against human leukemia and lymphoma cell lines. Among them, linalool showed the strongest activity against histiocytic lymphoma cells U937 (IC_{50}: 3.51 μg/ml, SI: 592.6) and Burkitt lymphoma cells P3HR1 (IC_{50}: 4.21 μg/ml, SI: 494.1). Ursolic acid was effective against P3HR1 cells (IC_{50}: 2.5 μg/ml, SI: 262.6) and chronic myelogenous leukemia cells K562 (IC_{50}: 17.79 μg/ml, SI: 36.91), whereas oleanolic acid inhibited the growth of P3HR1 cells (IC_{50}: 26.74 μg/ml, SI: 11.37). Luteolin exhibited effective activity against K562 cells ($IC_{50:}$ 18.96 μg/ml, SI: 5.14) and P3HR1 cells (IC_{50}: 18.99 μg/ml, SI: 5.13). We conclude that terpenes and flavonoid in commonly used medicinal plants possess strong activity against lymphoma and leukemia cells, especially human lymphoma cells, suggesting the potential use of these compounds for treatment of lymphoma.

Keywords: Antileukemia; *Plantago* genus; Terpenes; Flavonoid.

Introduction

Cancer accounted for 24% of all deaths in the United States and approximately 40% of the population will be diagnosed with cancer at some point in their lifetime. An estimated more

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than one million new cases of cancer were diagnosed, and more than 0.5 million cancer-related deaths occurred (Miller *et al*., 1992). Cancer is the leading cause of death in the developing and developed countries. Cancer claims over six million lives globally each year (Pezzuto, 1997). Many commonly used medicinal plants in Taiwan (Lin and Kan,1990) contain biologically active compounds of six classes such as benzoic compound (vanillic acid), flavonoid (luteolin), iridoid glycoside (aucubin), phenolic compounds (caffeic acid, chlorogenic acid, ferulic acid and p-coumaric acid), monoterpene (linalool), and triterpenes (oleanolic acid and ursolic acid) (Fig. 1,Table 1) (Duke, 1992; Samuelsen, 2000).

Caffeic acid was reported to inhibit proliferation of leukemia cells (Jian *et al*., 1999; Sakagami *et al*., 1995), whereas luteolin was effective against leukemia, melanoma and carcinomas of pancreas, ovary, brain, kidney, lung, colon and stomach (Matsukawa *et al*., 1993; Molnar *et al*., 1981; Pettit *et al*., 1996; Post and Varma, 1992; Ramanathan *et al*., 1994). Oleanolic acid was active against leukemia and carcinomas of lung, breast, pancreas, colon and kidney (Njoku *et al*., 1997; Noda *et al*., 1997; Umehara *et al*., 1992). Ursolic acid has been reported to inhibit the growth of leukemia, melanoma, hepatoma and carcinomas of lung, ovary, colon, cervix and brain (Ahn *et al*., 1998; Baek *et al*., 1997; Kim *et al*., 1999 and 2000; Lin *et al*., 1990).

In searching natural products for potent antileukemic activity, we evaluated the cytotoxic activity of ten pure compounds derived from commonly used medicinal plants in Taiwan using five different human leukemia and lymphoma cell lines.

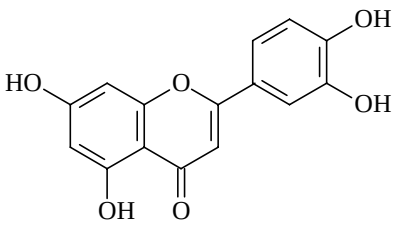
Table 1. Phytochemical Constituents of Common Medicinal Plants in Taiwan*

Medicinal Plant	Aucubin	Caffeic Acid	Chlorogenic Acid	p-Coumaric Acid	Ferulic Acid	Linalool	Luteolin	Oleanolic Acid	Ursolic Acid	Vanillic Acid
Allium sativum L.		+	+	+	+	+		+		
Artemisia dracunculus L.		+	+	+	+	+	+			+
Capsicum annuum L.		+	+	+		+				
Curcuma longa L.		+		+		+				+
Foeniculum vulgare Miller		+		+	+	+				+
Ocimum basilicum L.		+		+		+	+	+	+	
Plantago asiatica L.	+					+			+	
Plantago major L.	+	+	+	+	+		+	+	+	+
Scutellaria baicalensis Geo.										
Zingiber officinale Ros.		+	+	+	+	+				+
Ratio	2/10	8/10	5/10	8/10	5/10	8/10	3/10	3/10	3/10	5/10

* Cited from Duke (1992).

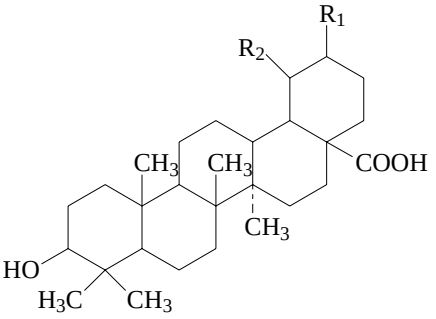
Chemicals structure

a. Flavonoid

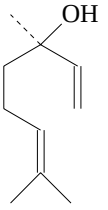


Luteolin

b. Terpenoids

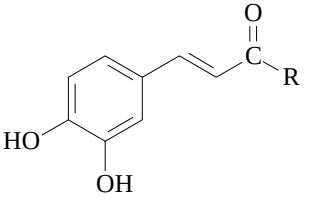


	R_1=	R_2=
Oleanolic acid	$-(CH_3)_2$	-H
Ursolic acid	$-CH_3$	$-CH_3$



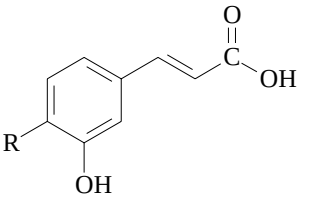
Linalool

c. Caffeic acid derivatives

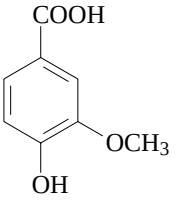


	R=
Caffeic acid	-OH
Chlorogenic acid	1,3,4,5-Tetrahydroxy-cyclohexane carboxylic acid

d. Other organic acids

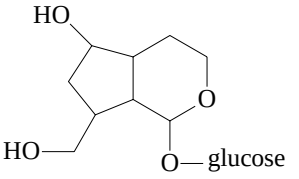


	R=
Ferulic acid	-OCH3
p-coumaric acid	-H



Vanillic acid

e. Iridoid glycoside



Aucubin

Figure 1. Chemical structures of natural products.

Materials and Methods

Pure Compounds

Aucubin, chlorogenic acid and luteolin were purchased from Wako Pure Chemical Industries, Ltd., (Japan). 5-Fluorouracil, caffeic acid, ferulic acid, oleanolic acid, p-coumaric acid, ursolic acid and vanillic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Linalool was purchased from Carl Roth (Chemische Fabrik, karisruhe, French). XTT (12,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-5-(phenylamino) carbonyl-2H-tetrarolium hydroxide) kits were purchased from Roche Diagnostics GmbH (Germany).

Culture of Human Leukemia and Lymphoma Cells

The promyelocytic leukemia cells HL-60 (ATCC CCL 240), chronic myelogenous leukemia cells K562 (ATCC CCL 243), acute lymphoblastic leukemia cells CCRF-CEM (ATCC CCL 119), histiocytic lymphoma cells U937 (ATCC CRL 1593) and Burkitt lymphoma cells P3HR1 (ATCC HTB 62) were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The normal human peripheral blood lymphocytes were supplied by the Blood Donor Center in Kaohsiung (Taiwan). Cells were grown in RPMI-1640 (Roswell Park Memorial Institute 1640) medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (GIBCO, Grand Island, New York).

Cancer Cell Cytotoxicity with XTT-based Colorimetric Assay

All human cancer cell lines were seeded onto 96-well plates with a concentration of 1.4×10^5 cells/ml and a volume of 90 μl per well. Different concentrations of pure compounds were applied to culture wells in triplicate. DMSO was used as negative control. After incubation for 3 days at 37°C with a 5% CO_2 incubator, the mixture of 0.1 ml PMS (Phenazine methosulfate: electron-coupling reagent) and 5 ml XTT were added to each well with a volume of 50 μl. The trays were incubated for 2 hours to allow XTT formazan production. The optical densities were determined with the ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 450 nm and a reference wavelength of 690 nm. Data were calculated as percentage of inhibition by the following formula: inhibition % = [100 – (ODt / ODs) × 100]%. ODt and ODs indicated the optical density of the test substances and the solvent control. The concentration of 50% cellular cytotoxicity of cancer cells (IC_{50}) of test substances were calculated (Chang *et al*., 2001).

Normal Cell Cytotoxicity

The normal human peripheral blood lymphocytes, supplied by the Blood Donor Center in Kaohsiung (Taiwan), were laid on Ficoll-Hypaque (Sigma-Aldrich), and centrifuged in 400 g at 25°C for 30 minutes. The cells at the interface were collected, washed three times,

and resuspended in RPMI 1640 medium supplemented with 10% FCS at a concentration of 2×10^6 cells/ml. The cytotoxicity test with XTT-based colorimetric assay and calculation of the concentration of 50% cytotoxicity (CC_{50}) were done as described previously (Chang *et al.*, 2001).

Statistical Analysis

The selectivity index (SI) was determined by the ratio of the CC_{50} to IC_{50}. The effects of the test compounds on inhibition of human cancer cell proliferation were statistically compared using the Student's t-test.

Results

Among the ten pure compounds tested, monoterpene (linalool), triterpenoids (oleanolic acid and ursolic acid) and flavonoid (luteolin) possessed the strongest antileukemic activity (Figs. 2 to 4). Linalool was found to possess a broad-spectrum of activity and the strongest activity against histiocytic lymphoma U937 cells (IC_{50}: 3.51 μg/ml) and Burkitt lymphoma P3HR1 cells (IC_{50}: 4.21 μg/ml). Ursolic acid exhibited potent antileukemic activity against P3HR1 cells (IC_{50}: 2.5 μg/ml) and chronic myelogenous leukemia K562 cells (IC_{50}: 17.79 μg/ml). The water soluble compounds (aucubin, caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid and vanillic acid) expressed a weak antileukemic activity (IC_{50}: 26–56 μg/ml).

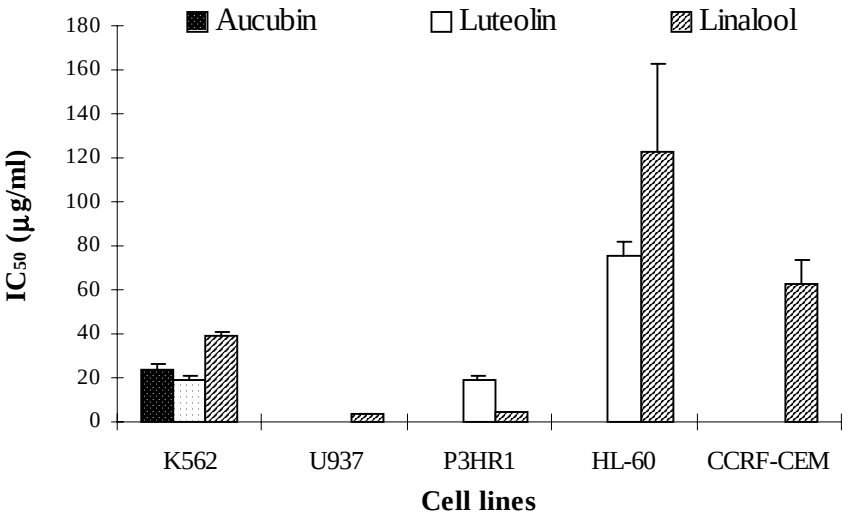


Figure 2. Antileukemic activity of aucubin, linalool and luteolin. Data were IC50 (the concentration of 50% cellular cytotoxicity of human tumor cells) of mean values obtained from three independent experiments and bars represented standard errors. Student's t-test was used to evaluate the p value and the mark indicated a significant difference between test and DMSO control ($^{*}p < 0.05$, $^{**}p < 0.01$).

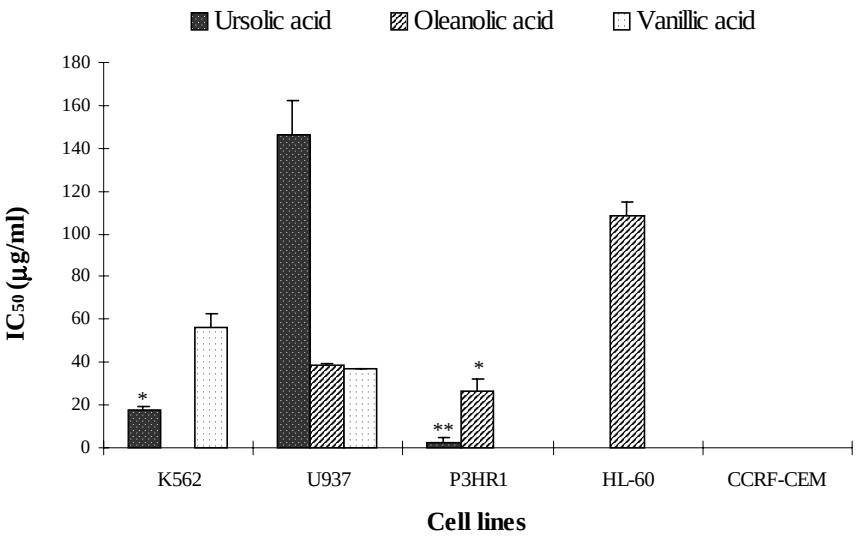


Figure 3. Antileukemic activity of triterpenes and vanillic acid. Data were IC50 (the concentration of 50% cellular cytotoxicity of human tumor cells) of mean values obtained from three independent experiments and bars represented standard errors. Student's t-test was used to evaluate the p value and the mark indicated a significant difference between test and DMSO control ($^{*}p < 0.05$, $^{**}p < 0.01$).

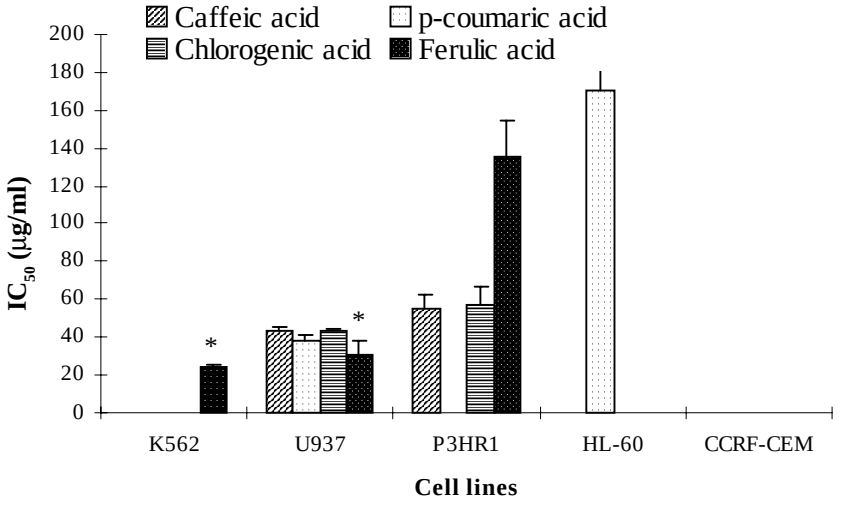


Figure 4. Antileukemic activity of phenolic compounds. Data were IC50 (the concentration of 50% cellular cytotoxicity of human tumor cells) of mean values obtained from three independent experiments and bars represented standard errors. Student's t-test was used to evaluate the p value and the mark indicated a significant difference between test and DMSO control ($^{*}p < 0.05$).

To evaluate whether test compounds affected human normal lymphocytes growth, human peripheral mononuclear cells were cultured with the test compounds or DMSO control. Results showed that the concentration (μg/ml) of 50% cytotoxicity (CC_{50}) against normal lymphocytes was in the order of: aucubin (44.68) < luteolin (97.50) < caffeic acid (109.13) < chlorogenic acid (111.52) < 5-fluorouracil (187.20) < vanillic acid (249.0) < ferulic acid (271.81) < p-coumaric acid (273.78) < oleanolic acid (304.0) < linalool (2080.0).

The assessments of antileukemic activity are shown in Tables 2 to 4. Results indicated that linalool possessed strong antileukemic activity, the order of activity against the various cell lines was U937 > P3HR1 > K562 > CCRF-CEM > HL-60 (SI = 592.6, 494.1, 52.75, 33.01 and 16.9, respectively). Flavonoid (luteolin) exhibited effective antileukemic activity against K562 cells (SI = 5.14) and P3HR1 cells (SI = 5.13) (Table 2). Triterpene (ursolic acid) showed a strong activity in inhibiting the proliferation of P3HR1 cells (SI = 262.6) and K562 cells (SI = 36.91) (Table 3). The water soluble phenolic compound (ferulic acid) exhibited a weak activity against the growth of K562 cells (SI = 11.07) and U937 cells (SI = 8.93) (Table 4).

Table 2. The Assessment of Antileukemic Activity of Aucubin, Linalool and Luteolin

Target Cell	5-FU*		Aucubin		Linalool		Luteolin	
	CC_{50}†	SI‡	CC_{50}	SI	CC_{50}	SI	CC_{50}	SI
Lymphocytes	187.2		44.7		2080.0		97.5	
K562		297.6		1.69		52.75		5.14
U937		249.6		§		592.6		§
P3HR1		195.0		§		494.1		5.13
HL-60		156.0		§		16.90		1.29
CCRF-CEM		317.3		§		33.01		§

*Control: positive control (5-fluorouracil, 5-FU), negative control (0.1% DMSO).
†The concentration (μg/ml) of 50% cytotoxicity against normal human lymphocytes.
‡ Selective index (SI) = CC_{50} / $IC_{50.}$
§SI < 1.0.

Table 3. The Assessment of Antileukemic Activity of Triterpenes and Vanillic Acid

Target Cell	5-FU*		Oleanolic Acid		Ursolic Acid		Vanillic Acid	
	CC_{50}†	SI‡	CC_{50}	SI	CC_{50}	SI	CC_{50}	SI
Lymphocytes	187.2		304.0		656.6		249.0	
K562		297.6		§		36.91		4.45
U937		249.6		7.86		4.49		6.75
P3HR1		195.0		11.37		262.6		§
HL-60		156.0		2.80		§		§
CCRF-CEM		317.3		§		§		§

*Control: positive control (5-fluorouracil, 5-FU), negative control (0.1% DMSO).
†The concentration (μg/ml) of 50% cytotoxicity against normal human lymphocytes.
‡Selective index (SI) = CC_{50} / $IC_{50.}$
§SI < 1.0.

Table 4. The Assessment of Antileukemic Activity of Phenolic Compounds

Target Cell	5-FU*		Caffeic Acid		Chlorogenic Acid		Ferulic Acid		p-Coumaric Acid	
	CC_{50}†	SI‡	CC_{50}	SI	CC_{50}	SI	CC_{50}	SI	CC_{50}	SI
Lymphocytes	187.2		109.1		111.5		271.8		237.8	
K562		297.6		§		§		11.07		§
U937		249.6		2.50		2.56		8.93		7.15
P3HR1		195.0		1.98		1.96		2.01		§
HL-60		156.0		§		§		§		1.60
CCRF-CEM		317.3		§		§		§		§

*Control: positive control (5-fluorouracil, 5-FU), negative control (0.1% DMSO).
†The concentration (μg/ml) of 50% cytotoxicity against normal human lymphocytes.
‡Selective index (SI) = CC_{50} / IC_{50}.
§SI < 1.0

Discussion

Many commonly used medicinal herbs in Taiwan have been shown to contain biologically active compounds of six classes such as benzoic compound (vanillic acid), flavonoid (luteolin), iridoid glycoside (aucubin), phenolic compounds (caffeic acid, p-coumaric acid, chlorogenic acid and ferulic acid) and triterpenes (oleanolic acid and ursolic acid) (Table 1) (Duke, 1992; Samuelsen, 2000). Luteolin was reported to inhibit proliferation of leukemia (P388, CEM, Raji, NK/LY), melanoma and carcinomas of pancreas, ovary, brain, kidney, lung, colon and stomach (Matsukawa *et al*., 1993; Molnar *et al*., 1981; Pettit *et al*., 1996; Post and Varma, 1992; Ramanathan *et al*., 1994). The present study showed a similar activity on cytotoxicity to Burkitt lymphoma (P3HR1) cells and chronic myelogenous leukemia (K562) cells (IC_{50} < 20 μg/ml, SI > 5.1) (Table 2).

Oleanolic acid was effective against leukemia (HL-60, M1) and carcinomas of lung, breast, pancreas, colon and kidney (Njoku *et al*., 1997; Noda *et al*., 1997; Umehara *et al*., 1992). In contrast to results reported by Umehara *et al.* (1992), oleanolic acid was found to possess a very weak activity against HL-60 cells (IC_{50} = 108.43 μg/ml), U937 cells (IC_{50} = 36.68 μg/ml) and P3HR1 cells (IC_{50} = 26.74 μg/ml) (Table 3). This discrepancy in results between the two different studies could be due to the difference in the number of target cells and the evaluating method used in the study. Ursolic acid has been reported to inhibit proliferation of leukemia (HL-60, K562, P388, L1210), melanoma, hepatoma and carcinomas of lung, ovary, colon, cervix and brain (Ahn *et al*., 1998; Baek *et al*., 1997; Kim *et al*., 1999 and 2000; Lin *et al*., 1990). In contrast to results reported by Baek *et al.* (1997), ursolic acid exhibited no activity against HL-60 cells, but possessed potent activity in inhibiting the proliferation of K562 cells (IC_{50} = 17.79 μg/ml, SI = 36.91) and P3HR1 cells (IC_{50} = 2.5 μg/ml, SI = 262.6) (Table 3). The discrimination in the results of ursolic acid between the two different studies could be due to the difference in the number of HL-60 cells and the evaluating method used in the study. To our knowledge, the antileukemic activities of monoterpene (linalool) have never been reported. In the present study, we have shown that linalool possessed potent activity against histiocytic lymphoma cells U937 and Burkitt lymphoma P3HR1 cells.

Among the water soluble pure compounds, caffeic acid was reported to inhibit proliferation of leukemia (HL-60, U937) cells (Jian *et al*., 1999; Sakagami *et al*., 1995). However, our results showed no activity against HL-60 cells, but possessed a very weak activity against U937 (IC_{50} = 43.71 μg/ml) and P3HR1 (IC_{50} = 55.21 μg/ml) (Table 3). The disagreement in anti-HL-60 results of caffeic acid between the two different studies could be due to the difference in the evaluating method and the number of target cells used in the study. Ferulic acid was effective in preventing mammary cancer (Shamon *et al*., 1994), but in this study it exhibited a weak cytotoxicity to K562 (IC_{50} = 24.56 μg/ml, SI = 11.07) and U937 (IC_{50} = 30.43 μg/ml, SI = 8.93) (Table 4). p-Coumaric acid possessed a weak activity against U937 cells (IC_{50} = 38.31 μg/ml, SI = 7.15) (Table 4).

Taken together, the present study has demonstrated that water insoluble pure compounds (monoterpene, triterpenes and flavonoid) possess strong antileukemic activities, especially linalool and human lymphoma cells, suggesting that they are both worth investigating *in vivo*.

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