

## The anti-cancer and anti-metastasis effects of phytochemical constituents from *Leucaena leucocephala*.

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### Abstract

The objective of this study was to evaluate the anti-cancer effects of compounds obtained from the plant *Leucaena leucocephala* in human chronic myelogenous leukaemia, prostate, liver, tongue, lung, melanoma, gastric and colon adenocarcinoma cancer cell lines. The data from the chemical analyses has identified 14 compounds belonging to prenols, sterol, flavonoids, coumarins and triterpene derivatives. The IC<sub>50</sub> values of pheophorbide a methyl ester, pyropheophorbide and pheophytin-a are 2.6, 3.69 and 1.89  $\mu$ M in AGS cell line. Pheophorbide a methyl ester, pyropheophorbide and pheophytin-a also displayed anti-migration and anti-invasion activity in AGS cell line. These results indicated that investigation on the anti-proliferation, anti-migration and anti-invasion effects of components from *Leucaena leucocephala*.

**Keywords:** *Leucaena leucocephala*, Anti-cancer, Mimosaceae.

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### Introduction

Cancer is the leading cause of death in the developed and developing countries. The potential anti-cancer agent from herb is an effective approach for the development of new drugs for treatment cancer patients [1-3]. *Leucaena leucocephala* is mimosoid tree also called white lead tree native to southern Mexico and northern Central America belonged to Mimosaceae family. The leaves and seeds of *Leucaena leucocephala* contain lipids, crude protein, tannin, carbohydrates, and mimosine [4]. Mimosine is a non-protein amino acid and the chemical structure is similar to tyrosine. Mimosine is toxic to animals but mimosine can be removed by soaking the leaves in water for 24 h [5]. However, mimosine has anticancer activity in human lung cancer cells by blocking cell cycle progression [6]. Previous study has shown that the extracts of the *Leucaena leucocephala* has anthelmintic, anti-oxidation and anti-diabetic activities [7-11]. The phytochemical data of *Leucaena leucocephala* revealed the presence of terpenes, flavonoids, coumarins and sterols [12]. In general, these components have many pharmacological activities in many studies.

The present work aims at isolating, screening and studying of these compounds from the leaves, seeds, green pods and brown pods of *Leucaena leucocephala* to evaluate their anti-cancer and pharmacological activity in different cancer cell lines.

### Materials and Methods

#### *Collection and identification of plant materials*

The specimen of *L. leucocephala* was collected from Pingtung County, Taiwan in December, 2008. A voucher specimen was characterized by Dr. Jin-Cheng Huang of Department of Forest Products Science and Furniture Engineering, National Chiayi University, Chiayi, Taiwan and deposited in the School of Medical and Health Sciences, Fooyin University, Kaohsiung County, Taiwan.

#### *Extraction and fractionation*

The air-dried seeds of *L. leucocephala* (5.0 kg) were extracted with MeOH (80 L  $\times$  6) at room temperature and the MeOH extract (162.5 g) was obtained upon concentration under reduced pressure. The MeOH extract was chromatographed

over silica gel (800 g, 70~230 mesh) using *n*-hexane/acetone as eluent to produce 5 fractions. Part of fraction 1 (6.11 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (40:1), enriched with acetone to furnish 10 fractions (1-1~1-10). Fraction 1-1 (1.72 g) was re-subjected to Si gel chromatography, eluting with *n*-hexane/acetone (80:1) to obtain linoleic acid (58 mg). Part of fraction 3 (6.94 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (50:1) to obtain  $\beta$ -sitosterol (15 mg). The air-dried brown pods of *L. leucocephala* (5.0 kg) were extracted with MeOH (80 L  $\times$  6) at room temperature and the MeOH extract (171.5 g) was obtained upon concentration under reduced pressure. The MeOH extract was chromatographed over silica gel using *n*-hexane/acetone as eluent to produce 6 fractions. Part of fraction 1 (7.02 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (40:1) to obtain  $\beta$ -sitostenone (6 mg). Part of fraction 2 (8.49 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (10:1), then enriched with acetone to furnish 8 fractions (2-1~2-8). Fraction 2-3 (2.33 g) was re-subjected to Si gel chromatography, eluting with *n*-hexane/acetone (40:1) to obtain lupeol (74 mg). Part of fraction 6 (13.89 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (4:1), then enriched with acetone to obtain 5  $\alpha$ , 8  $\alpha$ -epidioxy-(24  $\xi$ )-ergosta-6, 22-dien-3  $\beta$ -ol (43 mg). The air-dried green pods of *L. leucocephala* (5.0 kg) were extracted with MeOH (80 L  $\times$  6) at room temperature and the MeOH extract (132.5 g) was obtained upon concentration under reduced pressure. The MeOH extract was chromatographed over silica gel using *n*-hexane/acetone as eluent to produce 10 fractions. Part of fraction 2 (2.67 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (50:1) to obtain ficaprenol-11 (21 mg). Part of fraction 3 (6.77 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (8:1), then enriched with acetone to furnish 5 fractions (3-1~3-5). Fraction 3-3 (1.33 g) was further purified by another silica gel column using *n*-hexane/acetone to obtain squalene (5 mg). Part of fraction 5 (7.42 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (8:1) to obtain pheophorbide a methyl ester (12 mg). Part of fraction 6 (5.31 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (8:1) to obtain pyropheophorbide-a (16 mg). Part of fraction 8 (4.91 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (5:1) to obtain *trans*-coumaric acid and *cis*-coumaric acid mixture (8 mg). The air-dried leaves of *L. leucocephala* (5.0 kg) were extracted with MeOH (80 L  $\times$  6) at room temperature and the MeOH extract (159.5 g) was obtained upon concentration under reduced pressure. The MeOH extract was chromatographed over silica gel using *n*-hexane/acetone as eluent to produce 7 fractions. Part of fraction 1 (6.62 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (50:1) to obtain pheophytin-a (6 mg). Part of fraction 3 (7.56 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (8:1), then enriched with acetone to furnish 12 fractions (3-1~3-12). Fraction 3-7 (2.97 g) was re-subjected to Si gel chromatography, eluting with *n*-hexane/acetone (40:1) to obtain 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-S)-pheophytin-a (12

mg) and methyl-13<sup>2</sup>-hydroxy-(13<sup>2</sup>-S)-pheophorbide-b (9 mg). Part of fraction 6 (17.15 g) was subjected to Si gel chromatography by eluting with *n*-hexane/acetone (4:1) to obtain aristophyll-C (4 mg).

### **Cell lines and cell culture**

Eight human cancer cell lines were used in these studies. All the cell lines were obtained from Bioresource Collection and Research Center (BCRC, Taiwan). DU145 is human prostate cancer cell line and incubated in Eagle's Minimum Essential Medium. Hep G2 is human liver hepatocellular carcinoma and this cell line was incubated in Eagle's Minimum Essential Medium. SCC9 is oral squamous cell carcinoma and this cell line was incubated a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium. H441 is human adenocarcinoma of the lung and incubated in RPMI-1640 medium. AGS is human gastric cancer cell line, and the cell was incubated in F-12K Medium. WiDr is colorectal adenocarcinoma and incubated in Eagle's Minimum Essential Medium. K562 is human leukaemia cell line and incubated in Iscove's Modified Dulbecco's Medium. All cell lines were cultured in medium supplemented with 10% FBS (Gibco BRL) and antibiotic. These cell lines were routinely maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### **XTT assay**

Cells (1.0  $\times$  10<sup>4</sup>) were incubated with different concentrations of compounds in 200  $\mu$ l of medium in 96-well plate for 24 hr. Cell viability was determined by the XTT assay. The yellow tetrazolium salt XTT is reduced to a formazan dye by dehydrogenase enzymes in active cells. The formazan produced is proportional to active cells. In brief, a freshly prepared XTT-PMS labelling mixture (50  $\mu$ l) was added to the medium. The absorbance was measured at 450 nm.

### **Wound scratch migration assay**

Cell migration assay was performed using a scratch wound-healing format. The anti-migration of test compounds was further examined in AGS cell line. When the cell is confluent in a 6-well plate, cells were then scratched and treated with different concentrations of compounds. Cell migration was monitored by microscopy.

### **Matrigel invasion chamber assay**

The anti-invasion of test compounds was further examined in AGS cell line. Briefly, the upper sides of the transwell inserts (8  $\mu$ m pore size) were coated with Matrigel per insert in 24-well plates. 5  $\times$  10<sup>5</sup> cells were seeded in 500  $\mu$ L serum-free medium in each upper chamber. The bottom well were added with different concentrations of compounds in 500  $\mu$ L medium with 10% FBS. After incubation, the non-invasive cells were removed from the upper surface of the membrane with cotton swab, and the invasive cells on the under surface of the membrane. The under surface of the membrane were stained with Giemsa stain solution.

### Statistical analysis

All experiments were carried out at three times and at least triplicate. The results were expressed as the average of the mean values  $\pm$  Standard Deviation (SD). Analysis of the data was done with SigmaPlot software (Version 8.0, SPSS Scientific, Chicago, IL, USA) and SigmaStat (Version 2.03, SPSS Scientific) run on an IBM-compatible computer.

## Results

### Anti-proliferation of compounds in cancer cell lines

In the present study, we isolated 15 compounds from the different parts of *Leucaena leucocephala*. Linoleic acid and  $\beta$ -sitosterol were isolated from seeds. Lupeol,  $\beta$ -sitostenone and 5  $\alpha$ , 8  $\alpha$ -epidioxy-(24  $\xi$ )-ergosta-6, 22- dien-3  $\beta$ -ol from the brown pods. Pheophorbide a methyl ester, ficaprenol-11, squalene, mixture of *cis*- and *trans*-coumaric acid and pyropheophorbide-a were isolated from the green pods. Pheophytin-a, aistopyll-C, 13<sup>2</sup>-hydroxy-(13<sup>2</sup>-S)-pheophytin-a and methyl-13<sup>2</sup>- hydroxy-(13<sup>2</sup>-S)-pheophorbide b were isolated from the leaves. All compounds were tested in human chronic myelogenous leukaemia, prostate, liver, tongue, lung, melanoma, gastric and colon adenocarcinoma cancer cell lines. All compounds had no significant anticancer activity in human colon adenocarcinoma cell line (WiDr). Methyl-13<sup>2</sup>-hydroxy-

(13<sup>2</sup>-S)-pheophorbide b and pyropheophorbide displayed board anti-cancer activity in many cancer cell lines including Hep G2 (liver), A375 (melanoma), DU145 (prostate), SCC9 (tongue), H44 (lung) and AGS (stomach). Moreover, pheophorbide a methyl ester, pyropheophorbide and pheophytin-a had significant anti-proliferation activity in AGS cell line. The IC<sub>50</sub> values of compounds in different cancer cell lines were shown in Table 1.

### Pheophorbide a methyl ester, pyropheophorbide-a and pheophytin-a inhibit DNA synthesis, migration and invasion in AGS cell line

The chemical structure of pheophorbide a methyl ester, pyropheophorbide-a and pheophytin-a were shown in Figure 1. We next performed a BrdU incorporation assay to evaluate pheophorbide a methyl ester, pyropheophorbide and pheophytin-a DNA synthesis in AGS cell line. Pheophorbide a methyl ester, pyropheophorbide and pheophytin-a treatment for 24 h significantly decreased DNA synthesis in AGS cell line (Figure 2). We further examined the activity of anti-migration and anti-invasion in AGS cell line. The results indicated that the treatment of pheophorbide a methyl ester, pyropheophorbide-a and pheophytin-a for 24 h slightly inhibited migration and invasion (Figures 3A and 3B).

**Table 1.** Cytotoxicity of compounds obtained from *Leucaena leucocephala* in different cancer cell lines.

	IC <sub>50</sub> (μM)							
	DU145	Hep G2	SCC9	H441	A375	AGS	WiDr	K562
Linoleic acid	292.7 $\pm$ 10.32	>500.0	19.2 $\pm$ 1.40	2.4 $\pm$ 0.40	>500.0	92.1 $\pm$ 9.11	>500.0	16.3 $\pm$ 1.45
Pheophytin-a	6.6 $\pm$ 0.75	>500.0	5.8 $\pm$ 0.31	>500.0	35.3 $\pm$ 6.40	3.69 $\pm$ 0.42	>500.0	64.2 $\pm$ 5.30
Pheophorbide a methyl ester	81.2 $\pm$ 4.81	>500.0	6.4 $\pm$ 0.57	0.5 $\pm$ 0.02	>500.0	2.6 $\pm$ 0.43	>500.0	2.9 $\pm$ 0.32
13 <sup>2</sup> -Hydroxy-(13 <sup>2</sup> -S)-pheophytin-a	15.9 $\pm$ 1.17	>500.0	46.4 $\pm$ 5.30	>500.0	>500.0	140.5 $\pm$ 6.4	>500.0	0.7 $\pm$ 0.11
Aristophyll-C	29.3 $\pm$ 2.42	>500.0	1 $\pm$ 0.01	>500.0	>500.0	>500.0	440.8 $\pm$ 2.4	3.8 $\pm$ 0.78
Methyl-13 <sup>2</sup> -hydroxy-(13 <sup>2</sup> -S)-pheophorbide b	8.1 $\pm$ 1.00	157.8 $\pm$ 10.73	8.4 $\pm$ 0.78	68.4 $\pm$ 1.78	42.9 $\pm$ 5.40	9 $\pm$ 1.43	>500.0	36.6 $\pm$ 4.43
5 $\alpha$ , 8 $\alpha$ -epidioxy-(24 $\xi$ )-ergosta-6, 22- dien-3 $\beta$ -ol	191.5 $\pm$ 7.78	>500.0	13.5 $\pm$ 1.10	NDx	>500.0	8.1 $\pm$ 1.1	>500.0	2.8 $\pm$ 0.25
$\beta$ -sitosterol	218.5 $\pm$ 10.40	>500.0	14.2 $\pm$ 1.00	49.9 $\pm$ 4.32	>500.0	56.9 $\pm$ 7.22	>500.0	58.1 $\pm$ 5.32
Lupeol	126.3 $\pm$ 5.32	>500.0	143 $\pm$ 8.38	63.4 $\pm$ 7.41	>500.0	44.2 $\pm$ 4.40	>500.0	19.2 $\pm$ 3.11
$\beta$ -sitostenone	>500.0	>500.0	>500.0	0.018 $\pm$ 0.00	>500.0	>500.0	>500.0	>500.0
ficaprenol-11	>500.0	>500.0	436.2 $\pm$ 12.23	214.6 $\pm$ 9.34	>500.0	>500.0	>500.0	22.0 $\pm$ 2.4
Squalene	>500	>500.0	>500.0	>500.0	>500.0	>500.0	>500.0	14.1 $\pm$ 3.33

<i>cis</i> - and <i>trans</i> -coumaric acid	>500	>500.0	55.6 ± 7.41	392.1 10.42	± >500.0	>500.0	>500.0	199.0 ± 9.42
Pyropheophorbide-a	24.5 ± 3.23	327.7 ± 13.33	0.716 ± 0.10	2.3 ± 0.41	29.5 ± 3.41	1.89 ± 0.22	>500.0	0.5 ± 0.09
Cisplatin	1.8 ± 0.04	NDx	NDx	38.8 ± 2.78	96.5 ± 5.47	2.7 ± 0.11	>500.0	0.2 ± 0.02

\*ND: Not Determined. Values represent mean ± SD of three independent experiments.

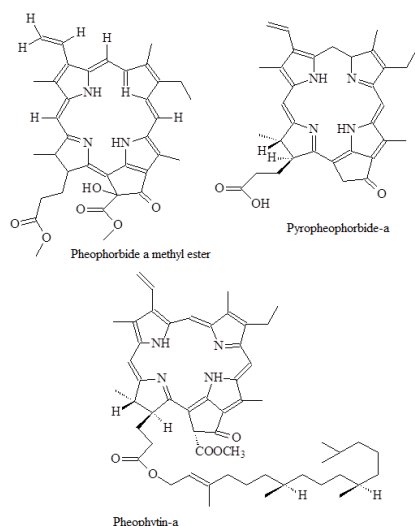


Figure 1. Structure of test chlorophylls.

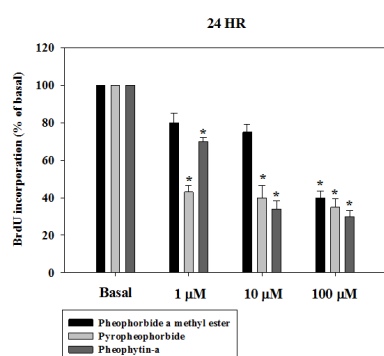


Figure 2. Effect of compounds on the BrdU incorporation of AGS cells. Values represent mean ± SD of three independent experiments. \*P < 0.05 vs. controls.

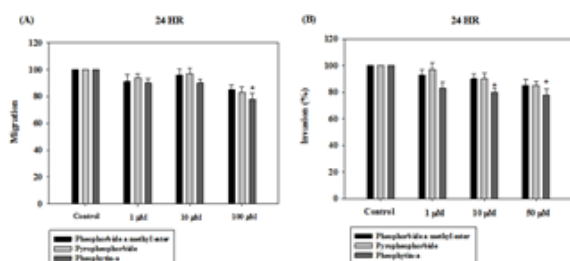


Figure 3. The effects of compounds on the invasion, and migration in AGS cells. Cells were treated with various concentrations of compounds and were then subjected to analyses for invasion, and migration. Values are expressed as mean ± SD of three independent experiments. \*P < 0.05 compared with the untreated control.

## Discussion

Plants have many pure compounds with biological activities. A research has indicated that *Leucaena leucocephala* possess flavonoids with anti-oxidation activity [12]. In the present study, we revealed the isolation and identification of 14 pure compounds from the different parts of *Leucaena leucocephala*. These compounds were also evaluated the anti-cancer activity. We further examined the activity of anti-migration and anti-invasion of pheophorbide a methyl ester, pyropheophorbide a and pheophytin-a in AGS cell line.

Photodynamic therapy is based on photosensitizers. Photosensitizers are light-sensitive molecules. Photoactivation produces peroxidative reactions and make cell damage and death [13,14]. Pheophorbide a methyl ester, pyropheophorbide a and pheophytin-a were previously isolated from *Phaeanthus ophthalmicus*, *Piper penangense* and *Zanthoxylum ailanthoides* Sieb. and Zucc [15-17]. These compounds are photosensitizers with anti-cancer activity. In the present study, we did not evaluate the phototoxicity of the compounds in AGS cell line.

Gastric cancer is the second leading cause of cancer mortality in the world [18]. Lymphatic metastasis is commonly observed in gastric cancer. Many factors are involved in gastric cancer progression, such as Epidermal Growth Factor Receptor (EGFR) and PI3K/Akt/mTOR pathway [19-22]. Metastasis is a complex progress and activates by many steps such as decreased adhesion, increased motility and invasion and so on. Migration is also key step across the metastatic cascade. In the present study, we have shown that pheophorbide a methyl ester, pyropheophorbide a and pheophytin-a not only inhibit cancer cell proliferation but also decrease invasion and migration in gastric cancer cell line. The detail *in vivo* mechanism of pharmacological activities can be examined in the future.

## Conclusion

The pure compounds were purified from the leaves, seeds, green and brown pods of *Leucaena leucocephala*. These compounds displayed important pharmacological activities such as anti-proliferation, anti-migration and anti-invasion in cancer cell lines. The information could provide a supportive role in the pharmaceutical field towards the development of new drugs.

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## Conflicts of Interest

The authors declare no conflict of interest.

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