

# Yellow and green pigments from *Calophyllum inophyllum* L. seed oil induce cell death in colon and lung cancer cells

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**Abstract.** Natural compounds have been candidates for anti-cancer medicine over the last 20 years. During the process of isolating seed oil from *Calophyllum inophyllum* L., yellow and green pigments containing multiple compounds with an aromatic structure were identified. High-performance liquid chromatography and nuclear magnetic resonance analysis of these pigments revealed that the compounds present were identical, but the concentration of the compounds was different. Treatment with the pigments was able to induce the death of DLD-1 human colon cancer cells and increase the percentage of the cells in the sub-G<sub>1</sub> and sub-G<sub>2</sub>/M phases in a dose-dependent manner. Additionally, the pigments were able to exhibit cytotoxic activity on A549 and H1975 human non-small cell lung cancer (NSCLC) cell lines at 24 h, with half-maximal inhibitory concentrations (IC<sub>50</sub>) values of 0.1206 and 0.0676%, respectively for green pigments, and 0.0434 and 0.0501%, respectively for yellow pigments. Furthermore, a decrease in IC<sub>50</sub> value was associated with an increase in the duration of treatment. However, a sharp decrease in IC<sub>50</sub> value of the yellow pigment was observed for H1975 cells at 48 h and for A549 cells at 72 h compared with no change in IC<sub>50</sub> value for the green pigment with time, suggesting that the pigments function and induce cell death differently in the two cell lines. An investigation was performed into the synergistic effect of the green pigment and gefitinib (Iressa®, ZD1839), which is a selective epidermal growth factor receptor-tyrosine kinase inhibitor to block growth factor-mediated cell proliferation. The combination of the green pigment and gefitinib resulted in an enhancement of the decrease in viability of A549 and

H1975 cells compared with treatment with gefitinib alone, which suggested that treatment with the green pigments was able to enhance the sensitivity of NSCLC cells to gefitinib. In conclusion, these pigments may be considered for development as anti-colon cancer agents.

## Introduction

*Calophyllum inophyllum* L. (*C. inophyllum*) of the family Clusiaceae is an associated mangrove species and is located in the tropical regions of Asia. In addition to its traditional use in treating skin problems, including rashes, ulcers, pimples, cuts, wounds and sores, the leaf extracts function as antioxidant, antidyslipidemic and anti-inflammatory agents (1,2). The seeds (tonka beans) of *C. cerasiferum* Vesque and *C. inophyllum* contain the highest levels of coumarins (2*H*-1-benzopyran-2-one) (3), which are phenolic substances composed of fused benzene and  $\alpha$ -pyrone rings that are secondary metabolites found in plants, bacteria and fungi. Environmental conditions and seasonal changes may affect the incidence of coumarins in various parts of the plant (4). Among coumarins, a number of compounds have been reported to exhibit anticancer properties, including imperatorin (5), esculetin (6) and osthole, which inhibits the migration and invasion of breast cancer cells, potentially via the inhibition of matrix metalloproteinase promoter and enzymatic activity (7). Another type of 4-phenylcoumarins isolated from *C. inophyllum* is calocoumarin A (8).

Colon cancer and small-cell and non-small-cell lung cancer (NSCLC) are important types of human cancer. Colon cancer arises in the colon or rectum due to the abnormal growth of cells with the ability to invade or spread to other tissue sites (9). In 2012, there were 1.4 million new cases of colon cancer and 694,000 associated mortalities from the disease (10). Therefore, it is necessary to develop novel strategies to increase the anticancer effects of colon cancer treatments. Lung cancer is considered to be the most common type of cancer in the world (11) and remains a major global health problem, accounting for 1.8 million annual mortalities worldwide in 2012 (12) and 17.8% of all cancer-associated mortalities in India in 2002 (13). Activation of the epidermal growth factor receptor (EGFR) signaling pathway in cancer

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cells was reported to be able to inhibit apoptosis and induce cell proliferation, angiogenesis and metastasis, leading to a poor disease prognosis (14).

The seed oil from *C. inophyllum* changes in color from yellow to green due to processing and storage conditions. The seed oil contains numerous compounds. The present study investigated the effects of the yellow and green pigments from the seed oil of *C. inophyllum* for the treatment of colon cancer and NSCLC in combination with gefitinib. These results may provide a rationale to utilize the pigments with or without gefitinib for the treatment of colon cancer and lung cancer.

## Materials and methods

**Chemicals and reagents.** Trifluoroacetic acid (TFA) and deuterated methanol were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The LiChrospher 100 RP-18e column (4.0 mm i.d. x250 mm; 5  $\mu$ m) was purchased from Merck KGaA. Methanol and acetonitrile were Liquid Chromatography LiChrosolv<sup>®</sup> and were purchased from Merck KGaA. High-performance liquid chromatography (HPLC) was performed using Waters 1525 binary HPLC pump, Waters in-line degasser AF, Waters 717 plus autosampler, and Waters 2487 dual  $\lambda$  absorbance detector (Waters Corporation, Milford, MA, USA). The proton nuclear magnetic resonance (<sup>1</sup>H-NMR) spectrum was evaluated using a Bruker Avance DRX500 instrument (Bruker Corporation, Billerica, MA, USA) at 500 MHz.

**Analysis of seed oil pigments from *C. inophyllum* L.** The seeds were collected from the coast of Chiayi County and heated to a range of temperatures from 70 to 105°C for at least 1 h to obtain green and yellow seed oil. The preliminary analysis indicated that, aside from fatty acids, the pigments were the major compounds in the seed oils, as determined by infrared spectrum analysis (Agilent 6890 N GC-FID; Agilent Technologies, Inc., Santa Clara, CA, USA). The pigment solution (10  $\mu$ l) was dissolved in 990  $\mu$ l absolute methanol. The solution was centrifuged at 9,400 x g for 10 min at room temperature. The supernatant was separated using a LiChrospher 100 RP-18e (4 mm i.d. x250 mm, 5  $\mu$ m; Merck KGaA) column with the following conditions: A mobile phase of 0.05% TFA-CH<sub>3</sub>CN (23:77), a flow rate of 1.0 ml/min and a column temperature maintained at 40°C and detected at 280 nm. An aliquot of sample (10  $\mu$ l) was dissolved in 600  $\mu$ l of methanol-d<sub>4</sub> completely and poured into the NMR tube. The <sup>1</sup>H-NMR spectrum was measured using a Bruker Avance DRX500 instrument (Bruker Corporation) at 500 MHz.

**Effects of the pigments on the DLD-1 human colon cancer cell line.** The DLD-1 human colon cancer cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). RPMI-1640 was obtained from Hyclone (GE Healthcare, Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Propidium iodide (PI), MTT, trypan blue and other chemicals were purchased from Sigma-Aldrich; Merck KGaA.

The DLD-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin G and

100  $\mu$ g/ml streptomycin. The cells were maintained at 37°C in humidified air containing 5% CO<sub>2</sub>. The culture medium was replaced every two days (15). The cells were plated in 60 mm culture dishes for all experiments. The culture medium was replaced with new medium when the cells reached 80% confluence, and then the cells were exposed to 0, 0.19, 0.38, 0.75, 1.50 and 3.00x10<sup>-3</sup>% of the yellow pigment and 0, 1.6, 3.1, 6.3, 12.5 and 25.0x10<sup>-3</sup>% of the green pigment for 24, 48 and 72 h at 37°C. Following treatment, the cells were treated with MTT solution to determine cell viability and propidium iodide solution to determine cell cycle.

Cell viability was evaluated by MTT assay, as previously described (16). The yellow MTT was reduced to purple formazan by dehydrogenase in the mitochondria of living cells. A solubilization agent, dimethyl sulfoxide (DMSO), was added to dissolve the insoluble purple formazan product and to form a colored solution. The absorbance of this colored solution can be quantified using a specific wavelength (550-600 nm) and a spectrophotometer. The cells (1x10<sup>6</sup>) were cultured in 60 mm tissue culture dishes at 37°C for 24 h. The culture medium was replaced with new medium, and the cells were exposed to various concentrations, as above, of the green or yellow pigments at 37°C for 24, 48 and 72 h. Following treatment, the cells were incubated with 0.5 mg/ml MTT for 2 h at room temperature and then lysed with DMSO at room temperature for 5 min. A 200  $\mu$ l aliquot of the resulting solution was then removed from the 60 mm culture dishes and transferred to 96-well plates. The absorbance of the solution in the 96-well plates was evaluated at 595 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The analyses of DNA damage and the cell cycle were performed by staining with PI and flow cytometry, as previously described (17,18). The cells (1x10<sup>6</sup>) were cultured in 60 mm tissue culture dishes at 37°C for 24 h. The RPMI 1640 culture medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) was replaced with fresh medium, and the cells were exposed to 0.60, 0.75 and 1.50x10<sup>-3</sup>% of the yellow pigment and 5.0, 12.5 and 25.0x10<sup>-3</sup>% of the green pigment at 37°C for 48 h. Subsequently, the cells were pooled, washed with phosphate-buffered saline (PBS), fixed in a PBS-methanol (volume/volume, 1:2) solution at room temperature for 5 min and then incubated at 4°C for at least 18 h. Following washing with PBS once, the cell pellets were stained with the PI solution supplemented with PBS, 40  $\mu$ g/ml PI and 40  $\mu$ g/ml of DNase-free RNase A (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature in the dark and then analyzed using a Becton-Dickinson FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A total of  $\geq$ 10,000 cells were counted per sample, and the DNA histograms were further evaluated using the Modfit software version no. 3.2 (BD Biosciences, Franklin Lakes, NJ, USA) on a PC workstation to determine the percentage of cells in various phases of the cell cycle and to quantify cells with DNA damage.

**Effects of the pigments on the A549 and H1975 human lung carcinoma cell lines.** The A549 and H1975 human lung carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and the cells were cultured at 37°C in a humidified atmosphere containing

5% CO<sub>2</sub> in RPMI-1640 complete medium supplemented with sodium bicarbonate (w/v, 2.2%), L-glutamine (w/v, 0.03%), penicillin (100 U/ml), streptomycin (100 µg/ml) and fetal calf serum (10%; Gibco; Thermo Fisher Scientific, Inc.). The cell lines were routinely analyzed to confirm that the cells were free of *Mycoplasma*. The cells were cultured at 5,000 cells per well in 96-well tissue culture plates at 37°C for 24 h in a humidified atmosphere with 5% CO<sub>2</sub>. To determine cell viability, 0.05, 0.10, 0.25, 0.50 and 1.00% of the green and yellow pigments of *C. inophyllum* alone or gefitinib (Iressa<sup>®</sup>, ZD1839; AstraZeneca, London, UK) in combination with 0.00 and 0.05% green pigment, respectively, were introduced to the cells following plating. At the end of the culture period, 20 µl MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added per well, and the cells were incubated for an additional 2 h at 37°C. The absorbance was then evaluated at 490 nm using an ELISA plate reader (Bio-Rad Laboratories, Inc.).

**Statistical analyses.** For each protocol, three to four independent experiments were performed. The results are presented as the mean ± standard deviation. The statistical analyses were performed using the SigmaPlot 2000 software version 11.0 (Systat Software, San Jose, CA, USA). The differences in the evaluated variables between the experimental and control groups were assessed by the unpaired t-test, and comparisons between multiple groups were assessed using two-way analysis of variance and Tukey's honest significant difference test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Differences in compounds contained in yellow and green pigments obtained from seed oil of *C. inophyllum*.** The green and yellow pigments were obtained from the same batch of seeds using different processing methods. The present study hypothesized that both pigments contained identical compounds but with differing concentrations. Therefore, the pigments were dissolved in methanol and the nuclear magnetic resonance spectra were evaluated. The results demonstrated that the pigments contained similar aromatic protons at 7.0-7.5 ppm (Fig. 1). HPLC analysis at 280 nm indicated multiple types of aromatic compounds in the pigments. The green and yellow pigments exhibited identical banding patterns (Fig. 2). The analysis of 9 major compounds determined that the concentrations were the same for peaks 8 and 9 in green and yellow pigments. The concentrations were higher for peaks 4 and 5 in the yellow pigment compared with the same peaks in the green pigment. By contrast, the concentrations of the remaining 7 peaks were higher in the green pigment compared with the yellow pigment (Table I). Due to these differences, the present study evaluated the effects of the yellow and green pigments on two major cancer cell types, including the DLD-1 human colon carcinoma cell line and the A549 and H1975 human NSCLC cell lines.

**Yellow and green pigments inhibit the viability of DLD-1 cells.** The treatment of the DLD-1 human colon carcinoma cell line with the green pigment at concentrations of 0, 5,

12.5 or 25x10<sup>-3</sup>% or the yellow pigment at concentrations of 0, 0.6, 0.75, 1.5 or 3.0x10<sup>-3</sup>% for 24, 48 and 72 h resulted in a dose-dependent decrease in cell viability with both pigments as determined by the MTT assay (Fig. 3). The yellow and green pigments differed in their effects on cell viability. Following 48 and 72 h, there was a 40% decrease in cell viability as compared with the untreated cells when treated with the green pigment at a concentration of 12.5x10<sup>-3</sup>%, whereas there was a 30% decrease in cell viability as compared with the untreated cells when treated with the green pigment at a concentration of 1.5x10<sup>-3</sup>%.

**Treatment with pigments from seed oil of *C. inophyllum* induces apoptosis and G<sub>2</sub>/M cell cycle arrest in DLD-1 cells.** To evaluate whether the green or yellow pigments are able to induce apoptosis or arrest cell cycle, the DLD-1 cells were exposed to the green pigment at concentrations 0, 5, 12.5 and 25x10<sup>-3</sup>% or the yellow pigment at concentrations 0, 0.6, 0.75, 1.5 and 3.0x10<sup>-3</sup>% for 24 h. The fraction of cells associated with apoptosis and DNA damage (sub-G<sub>1</sub>) was evaluated by PI staining and flow cytometry. DLD-1 is a colorectal adenocarcinoma cell line, which was isolated by D.L. Dexter and associates during 1977-1979 (19). DLD-1 cells are positive for p53 antigen expression. DLD-1 cells also express metastatic and invasive characteristics, and may be transplanted into nude mice. For these reasons, the DLD-1 cells were selected in the present study.

As presented in Fig. 4A, treatment with the yellow pigment was able to induce an increased fraction of DLD-1 cells in the sub-G<sub>1</sub> phase (increased to 16% with 1.5x10<sup>-3</sup>%, compared with 0.6% in the untreated cells). Treatment with the green pigment was able to induce a sharp increase of DLD-1 cells in the sub-G<sub>1</sub> phase (2 to 57% with 5x10<sup>-3</sup> and 25x10<sup>-3</sup>% green pigment, respectively, as compared with 0.3% in the untreated cells) at 37°C for 24 h treatments.

Although the portion of cells in the G<sub>1</sub> and S cell cycle phases did not change significantly when treated with the three different concentrations of yellow (0.6x10<sup>-3</sup>, 0.75x10<sup>-3</sup> and 1.5x10<sup>-3</sup>%) and green pigments (5x10<sup>-3</sup>, 12.5x10<sup>-3</sup> and 2.5x10<sup>-3</sup>%), the fraction of cells in the G<sub>2</sub>/M cell cycle phase was increased in a dose-dependent manner (16 to 27% with 0.6x10<sup>-3</sup> and 1.5x10<sup>-3</sup>% yellow pigment; Fig. 4B).

However, treatment with yellow pigment induced a different phase change. The fraction of cells in the G<sub>1</sub> and S phases decreased when the cells were treated with 0.75x10<sup>-3</sup>% and 1.5x10<sup>-3</sup>% of yellow pigment, but the fraction of cells in the G<sub>2</sub>/M phase increased in a dose-dependent manner.

**Yellow and green pigments inhibit the survival of the NSCLC cells.** To investigate whether the pigments are able to exhibit any cytotoxic effects on human NSCLC cells, two different NSCLC cell types, A549 bronchioloalveolar cell carcinoma and H1975 adenocarcinoma cells, were employed in the present study. The cells were treated with various concentrations (0.05, 0.10, 0.25, 0.50 and 1.00%) of the pigments for 24-72 h, and the cell viability was then assessed by MTS assay. In A549 and H1975 cells, treatment with yellow and green pigments induced a concentration-dependent reduction in cell viability (Figs. 5 and 6). The level of cytotoxicity induced by the green and yellow pigments was determined by IC<sub>50</sub> value

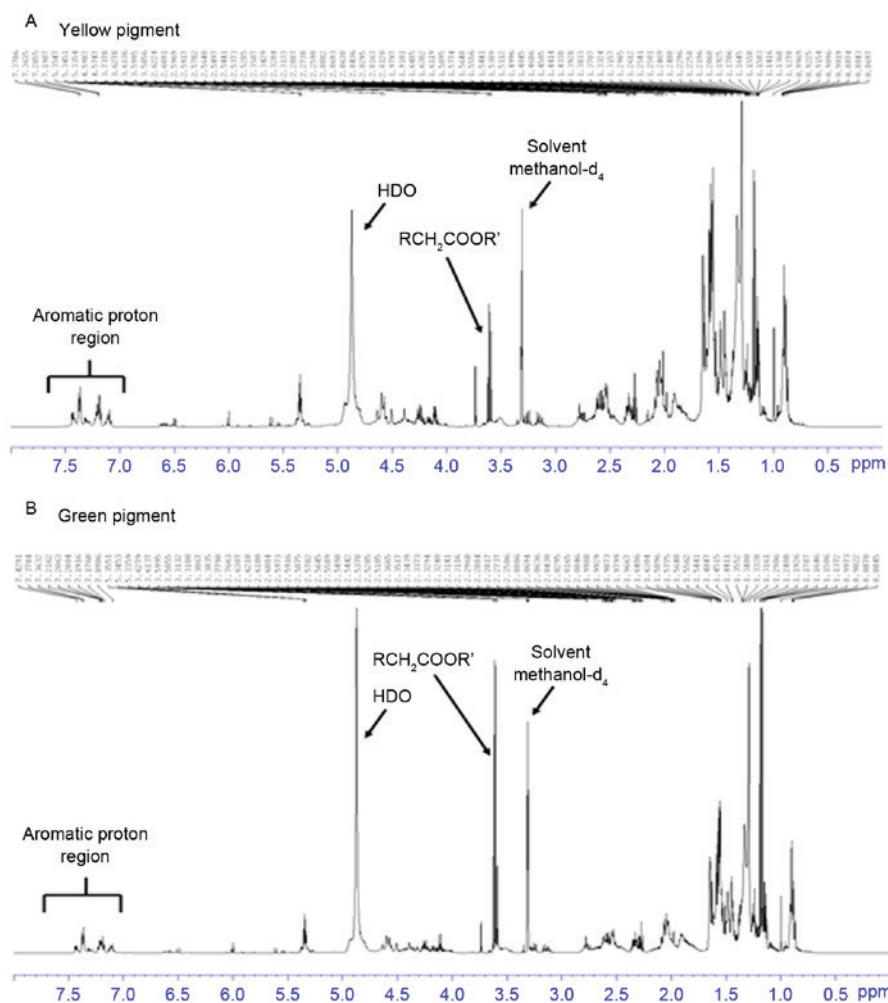


Figure 1. Nuclear magnetic resonance analysis of (A) yellow and (B) green pigments from the seed oil obtained from *Calophyllum inophyllum* L. ppm, parts per million.

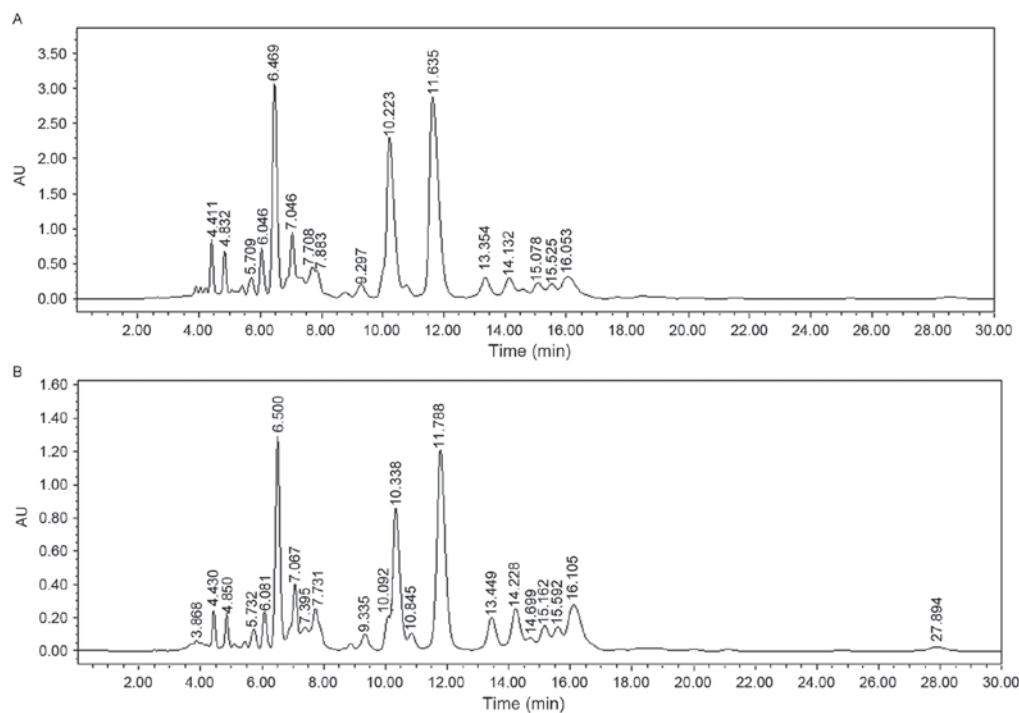


Figure 2. High-performance liquid chromatography analysis of (A) yellow and (B) green pigments from the seed oil obtained from *Calophyllum inophyllum* L. AU, absorption units.



Table I. Differences in the concentration of nine major compounds between the yellow and green pigments.

Item	Major peak	Yellow pigment			Green pigment		
		RT	Area	Area (%)	RT	Area	Area (%)
1	8	6.469	31937683	16.26	6.500	13641377	15.74
2	9	7.046	11713133	5.96	7.067	5735710	6.62
3	11	7.708	7969980	4.06	7.731	5522168	6.37
4	13	10.223	42321622	21.54	10.338	14476792	16.71
5	14	11.635	60684041	30.89	11.788	24102810	27.82
6	15	13.354	6879517	3.50	13.449	4574100	5.28
7	16	14.132	6423519	3.27	14.228	5804760	6.70
8	18	15.078	4736314	2.41	15.162	3212602	3.71
9	20	16.053	11111070	5.66	16.105	9580900	11.06

RT, retention time.

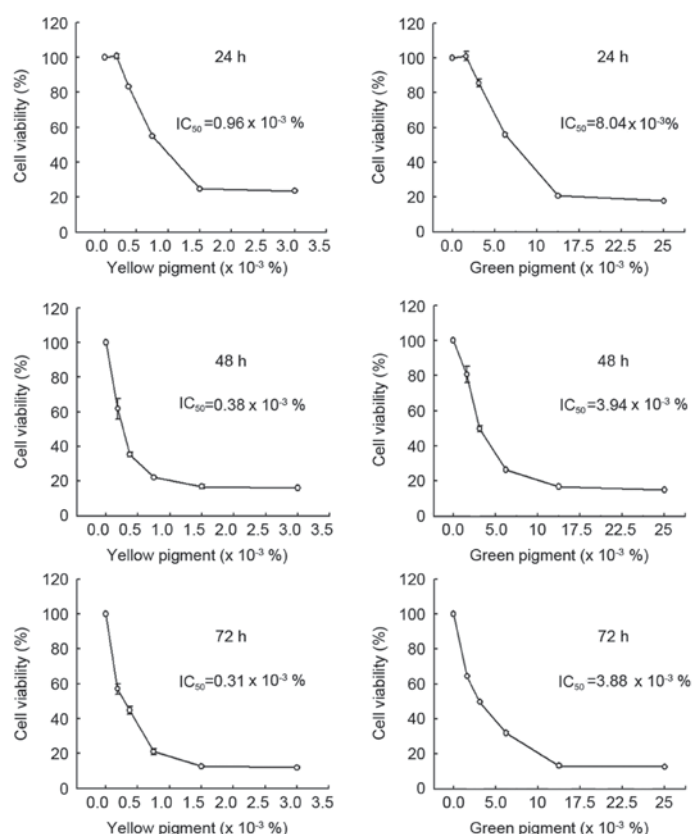


Figure 3. Cell viability analysis in green or yellow pigment-treated DLD-1 cells. DLD-1 cells were plated in 60 mm culture dishes at 80% confluence and treated with the indicated concentrations of pigment for 24 h. Following treatment, the MTT reagent (0.5 mg/ml) was added to the cells for 2 h at 37°C, and the cells were lysed with dimethyl sulfoxide. Absorbance was evaluated at 595 nm. IC<sub>50</sub>, half-maximal inhibitory concentration.

at 24 h. The green pigment induced increased cell death in the A549 cells compared with the H1975 cells, with IC<sub>50</sub> values of 0.1206 and 0.0676%, respectively (Fig. 5). Treatment with the yellow pigments exhibited increased cytotoxicity against H1975 and A549 cells at 24 h, with lower IC<sub>50</sub> values of 0.0434 and 0.0501%, respectively (Fig. 6).

*Treatment with green pigments increases the cytotoxic effect of gefitinib.* The present study aimed to determine whether the

green pigments are able to increase the cytotoxic effects of gefitinib in NSCLC cells. The effect of combined treatment of green pigments and gefitinib on cell viability was investigated by MTS assay. Treatment with gefitinib alone was able to reduce the viability of A549 and H1975 cells to a similar extent and decrease cell viability with increasing concentrations from 2.5 to 5  $\mu$ M (Table II). By contrast, combined treatment with green pigments and gefitinib for 24 h resulted in a greater decrease in viability in the H1975 cells compared

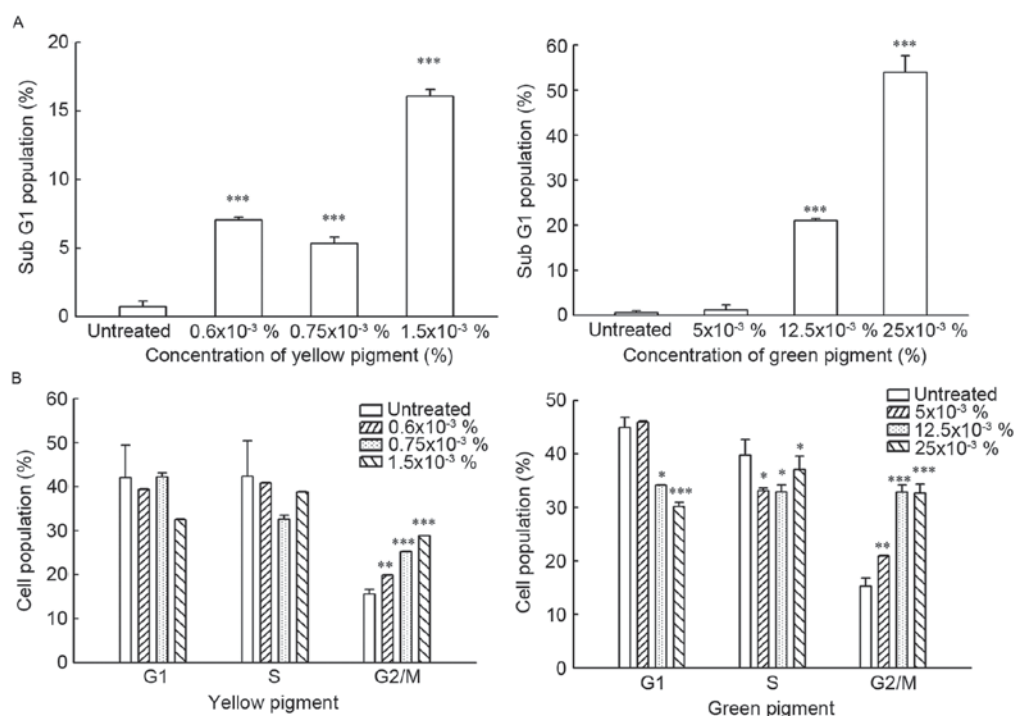


Figure 4. (A) Apoptotic analysis of green or yellow pigment-treated DLD-1 cells. \*\*\* $P < 0.001$  vs. untreated. (B) Cell cycle analysis of green or yellow pigment-treated DLD-1 cells. DLD-1 cells were plated in 60 mm culture dishes at 80% confluence and treated with the indicated concentrations of pigment for 24 h. Following treatment, the cells were harvested and fixed in phosphate-buffered saline-methanol (v/v, 1:2) solution. The cells were stained with propidium iodide followed by flow cytometric analysis. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. untreated.

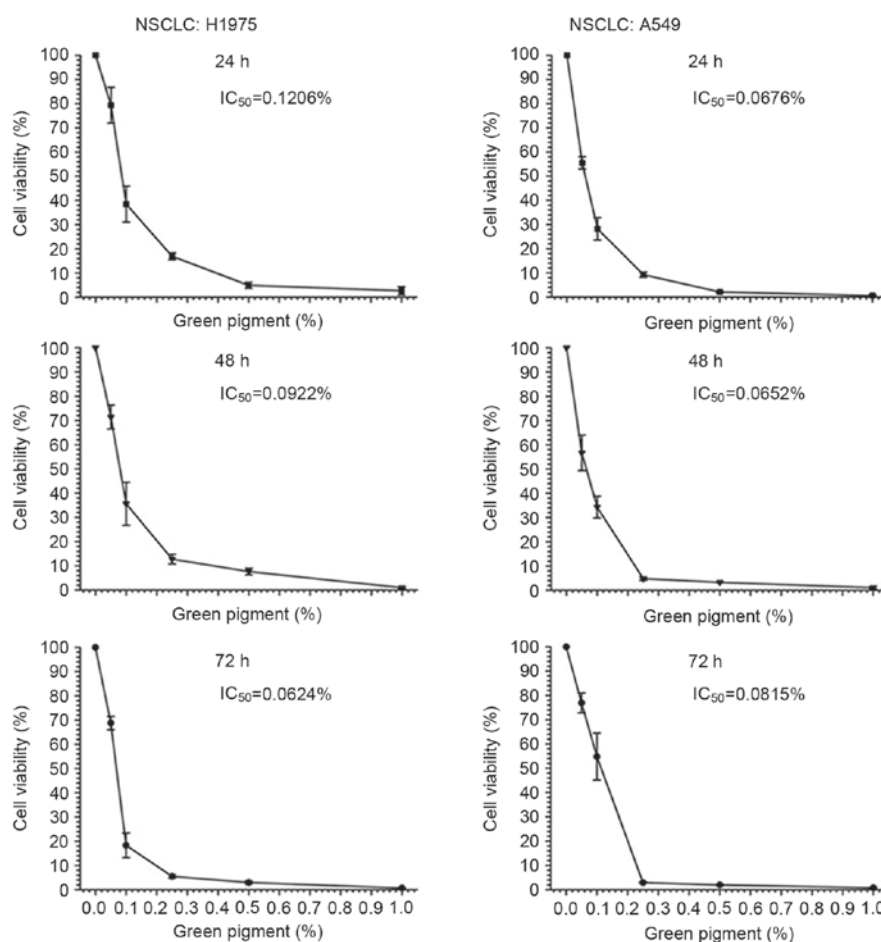


Figure 5. Green pigment induces cytotoxicity in human lung cancer cells. Various concentrations (0.05, 0.10, 0.25, 0.50 and 1.00%) of the pigment were added to A549 or H1975 cells for 24-72 h. Cell viability was determined by MTS assay. NSCLC, non-small cell lung cancer.

Table II. Co-treatment of green pigments and gefitinib increases cytotoxicity.

## A, A549 cells

Gefitinib ( $\mu\text{M}$ )	Green pigment		Probability	Differences in cell viability between 0-0.05% green pigment
	0% <sup>a</sup>	0.05% <sup>b-d</sup>		
0	100	58.02 $\pm$ 1.14	P<0.001	41.98 $\pm$ 1.03 <sup>a</sup>
1	92.03 $\pm$ 1.52	50.80 $\pm$ 1.56	P=0.022	41.23 $\pm$ 1.24 <sup>a</sup>
2.5	88.65 $\pm$ 1.00	48.28 $\pm$ 2.00	P=0.007	40.37 $\pm$ 1.45 <sup>a</sup>
5	75.23 $\pm$ 1.20	46.13 $\pm$ 1.58	P=0.021	29.10 $\pm$ 1.41 <sup>a</sup>
10	68.05 $\pm$ 2.00	31.14 $\pm$ 0.98	P<0.001	36.91 $\pm$ 1.17 <sup>a</sup>

## B, H1975 cells

Gefitinib ( $\mu\text{M}$ )	Green pigment		Probability	Differences in cell viability between 0-0.05% green pigment
	0% <sup>a</sup>	0.05% <sup>b-d</sup>		
0	100 $\pm$ 0	57.61 $\pm$ 2.00	P<0.001	42.39 $\pm$ 1.11 <sup>a</sup>
1	94.55 $\pm$ 1.23	43.03 $\pm$ 1.25	P=0.002	51.52 $\pm$ 1.16 <sup>a</sup>
2.5	89.12 $\pm$ 2.10	42.75 $\pm$ 1.77	P=0.003	46.37 $\pm$ 1.24 <sup>a</sup>
5	76.23 $\pm$ 0.56	39.08 $\pm$ 1.20	P<0.001	37.15 $\pm$ 1.35 <sup>a</sup>
10	69.00 $\pm$ 1.78	32.72 $\pm$ 0.56	P<0.001	36.28 $\pm$ 1.74 <sup>a</sup>

Gefitinib (1, 2.5, 5 and 10  $\mu\text{M}$ ) and/or green pigments (0.05%) were added to A549 or H1975 cells for 24 h. Cell viability was determined by MTS assay. The comparison between cells treated with drug alone or with a combination of gefitinib and IR pigment was analyzed using Tukey's honestly significant difference test. <sup>a</sup>P<0.05, 1 and 2.5 vs. 5 and 10  $\mu\text{M}$  gefitinib; <sup>b</sup>P<0.05, 1, 2.5 and 5 vs. 0  $\mu\text{M}$  gefitinib; <sup>c</sup>P<0.05, 10 vs. 0  $\mu\text{M}$  gefitinib; <sup>d</sup>P<0.05, 2.5 and 5 vs. 10  $\mu\text{M}$  gefitinib.

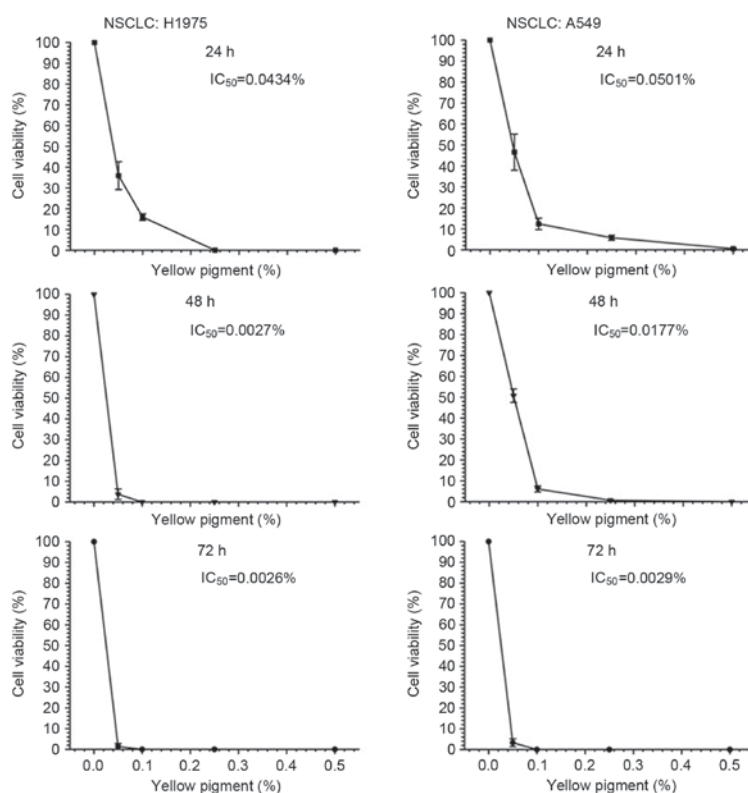


Figure 6. Yellow pigment induces cytotoxicity in human lung cancer cells. Various concentrations (0.05, 0.10, 0.25, 0.50 and 1.00%) of the pigments were added to A549 or H1975 cells for 24-72 h. Cell viability was determined by MTS assay. NSCLC, non-small cell lung cancer.

with the A549 cells at concentrations 1 to 5  $\mu$ M. Compared with the treatment with 0.05% green pigment alone, the treatment with 1  $\mu$ M gefitinib and 0.05% green pigment was able to markedly decrease cell viability by 14.6% (from 57.6 to 43.0%) for the H1975 cells and 7.22% (from 58.02 to 50.80%) for the A549 cells, with a difference in viability of 51.52 and 41.23% between cells treated with or without gefitinib for H1975 and A549 cells, respectively.

## Discussion

Natural medicines have been a target for the development of cancer drugs over the last 20 years. The leaf and seed extracts of *C. inophyllum* have been revealed to exhibit anti-inflammatory and antioxidant functions (1,2). The seed oil has been demonstrated to promote wound healing and to exhibit antibacterial activity (8). Furthermore, coumarins are present in the seed extracts, with calophyllolide 3 as the major compound along with inophyllum B, C, P and E (3). A number of coumarins have been revealed to have anticancer activities (5-7). The present study did not purify the compounds and instead used the crude extracts from the seed oil, which differed in color. The HPLC analysis demonstrated no differences in the identity of the nine major compounds present, with major distinction between the green and yellow pigments being the difference in concentrations (Fig. 2 and Table I), implying a possible change in the structure of the yellow and green pigments occurring during the isolation process. Therefore, the present study evaluated whether the pigments function differently in their treatment of cancer cells.

Colon cancer and lung cancer have been the most prevalent types of cancer worldwide over the last 15 years (10,11). Despite the observation that both pigments are able to induce the apoptosis of the DLD-1 colon cancer cells and increase the fraction of cells in the G<sub>2</sub>/M phase in a concentration-dependent manner (Fig. 4), the yellow pigments were more toxic with a ~10-fold concentration difference compared with the green pigment (Fig. 3). The difference in the concentrations required of each pigment for the same level of toxicity indicated that the pigments differed in the induction of apoptosis of DLD-1 colon cancer cells (Fig. 3). Numerous anticancer agents function via the induction of apoptosis (20). However, these anticancer agents may also trigger cell cycle checkpoints, which induce cell cycle arrest and thus protects cells from apoptosis (21,22). As malignant cells often have defects at the G<sub>1</sub> checkpoint, a common strategy of anticancer treatment is to activate the G<sub>2</sub> checkpoint in cancer cells. Therefore, the G<sub>2</sub> checkpoint represents a survival mechanism for cancer cells to avoid therapy-induced apoptosis (23). Numerous pigments that are extracted from plants exert anticancer effects. For example, curcumin, a naturally occurring yellow pigment isolated from the turmeric plant, is able to induce apoptosis in various cancer cell lines (24). Wang *et al.* (25) demonstrated that curcumin treatment was able to induce the activation of the checkpoint kinase 1-mediated G<sub>2</sub> checkpoint, which was associated with the induction of G<sub>2</sub>/M cell cycle arrest and the resistance of hepatoma cells to curcumin-induced apoptosis. These findings are also consistent with the present study, where the yellow and green pigments were revealed

to potentiate the effects of chemotherapy on DLD-1 colon cancer cells via G<sub>2</sub>/M cell cycle arrest and the induction of apoptosis.

Different types of cancer cells differ in their response to treatment with gefitinib (26). Subsequently, the present study investigated the effects of the pigments on A549 and H1975 NSCLC cells, respectively. With regards to the treatment of NSCLC, gefitinib, a small molecule EGFR tyrosine kinase inhibitor, has been used to prolong the survival of patients with advanced NSCLC following first- and second-line chemotherapies (26). A previous study examined the effect of NKG2-D-activating natural killer cell receptor expression in NSCLC cells, and revealed that gefitinib attenuated NK cell-mediated cell death (27). However, the use of gefitinib may induce gefitinib-resistant NSCLC via the overexpression of cathepsin L (28). Therefore, the combination of gefitinib with other drugs may increase the killing of NSCLC cells. Recently, a synergistic effect of high concentrations of gefitinib and celecoxib on A549 cells significantly increased the anti-proliferative and pro-apoptotic effects of the drugs by downregulation of cyclooxygenase-2 and phosphorylated-EGFR (29).

The synergistic effect of gefitinib and 0.05% green pigments was investigated in the present study. Green pigment was selected for this part of the study as a lower cytotoxicity of green pigments was observed in A549 and H1975 cell lines compared with yellow pigments. Treatment with green pigments alone or in combination with gefitinib was able to increase the cell death of A549 and H1975 NSCLC cell lines (Table II). A synergistic effect was observed with 10  $\mu$ M gefitinib and 0.05% green pigments for A549 cells, and with 1  $\mu$ M gefitinib and 0.05% green pigments for H1975 cells, indicating that the two cell lines differed in their response to the combination treatment of gefitinib and the green pigments. In conclusion, treatment with yellow and green pigments was able to increase the rate of apoptosis and the percentage of colon cancer cells in the sub-G<sub>2</sub>/M phase. It was also indicated that the yellow pigment was more toxic to the colon cancer cells and the NSCLC cells compared with the green pigment. Variable synergistic effects of gefitinib and the green pigment were observed in the two NSCLC cell lines (A549 and H1975).

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## References

1. Prasad J, Shrivastava A, Khanna AK, Bhatia G, Awasthi SK and Narendera T: Antidyslipidemic and antioxidant activity of the constituents isolated from the leaves of *Calophyllum inophyllum*. *Phytomedicine* 19: 1245-1249, 2012.
2. Tsai SC, Liang YH, Chiang JH, Liu FC, Lin WH, Chang SJ, Lin WY, Wu CH and Weng JR: Anti-inflammatory effects of *Calophyllum inophyllum* L. in RAW264.7 cells. *Oncol Rep* 28: 1096-1102, 2012.
3. Spino C, Dodier M and Sotheeswaran S: Anti-HIV coumarins from calophyllum seed oil. *Bioorg Med Chem Lett* 8: 3475-3478, 1998.



4. Venugopala KN, Rashmi V and Odhav B: Review on natural coumarin lead compounds for their pharmacological activity. *Biomed Res Int* 2013: 963248, 2013.
5. Luo KW, Sun JG, Chan JY, Yang L, Wu SH, Fung KP and Liu FY: Anticancer effects of imperatorin isolated from *Angelica dahurica*: Induction of apoptosis in HepG2 cells through both death-receptor and mitochondria-mediated pathways. *Chemotherapy* 57: 449-459, 2011.
6. Yun ES, Park SS, Shin HC, Choi YH, Kim WJ and Moon SK: p38 MAPK activation is required for esculetin-induced inhibition of vascular smooth muscle cells proliferation. *Toxicol In Vitro* 25: 1335-1342, 2011.
7. Yang D, Gu T, Wang T, Tang Q and Ma C: Effects of osthole on migration and invasion in breast cancer cells. *Biosci Biotechnol Biochem* 74: 1430-1434, 2010.
8. Itoigawa M, Ito C, Tan HT, Kuchide M, Tokuda H, Nishino H and Furukawa H: Cancer chemopreventive agents, 4-phenylcoumarins from *Calophyllum inophyllum*. *Cancer Lett* 169: 15-19, 2001.
9. Cooper GM: The Development and Causes of Cancer. The Cell: A Molecular Approach. 2nd edition. Sinauer Associates, Sunderland, MA, 2000.
10. World Health Organization: Cancer worldwide. In: World Cancer Report 2014. Stewart BW and Wild CP (eds). IARC Nonserial Publication, pp16-81, 2014.
11. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. *CA Cancer J Clin* 55: 74-108, 2005.
12. World Health Organization: GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012. Cancer fact sheets: Lung. [http://globocan.iarc.fr/Pages/fact\\_sheets\\_cancer.aspx](http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). Accessed Dec 27, 2013.
13. Dikshit R, Gupta PC, Ramasundarahettige C, Gajalakshmi V, Aleksandrowicz L, Badwe R, Kumar R, Roy S, Suraweera W, Bray F, *et al*: Cancer mortality in India: A nationally representative survey. *Lancet* 379: 1807-1816, 2012.
14. Earp HS, Dawson TL, Li X and Yu H: Heterodimerization and functional interaction between EGF receptor family members: A new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat* 35: 115-132, 1995.
15. Yeh CH, Yang ST and Chen CH: Calvatia lilacina protein extract induces apoptosis through endoplasmic reticulum stress in human colon carcinoma cells. *Process Biochem* 46: 1599-1606, 2011.
16. Chen CH, Lin WC, Kuo CN and Lu FJ: Role of redox signaling regulation in propyl gallate-induced apoptosis of human leukemia cells. *Food Chem Toxicol* 49: 494-501, 2011.
17. Yang JT, Li ZL, Wu JY, Lu FJ and Chen CH: An oxidative stress mechanism of shikonin in human glioma cells. *PLoS One* 9: e94180, 2014.
18. Chen CY, Li ZL, Chung KT, Lu FJ and Chen CH: Liriodenine enhances the apoptosis effect of valproic acid in human colon cancer cells through oxidative stress upregulation and Akt inhibition. *Process Biochem* 49: 1990-2000, 2014.
19. Chen TR, Dorotinsky CS, McGuire LJ, Macy ML and Hay RJ: DLD-1 and HCT-15 cell lines derived separately from colorectal carcinomas have totally different chromosome changes but the same genetic origin. *Cancer Genet Cytogenet* 81: 103-108, 1995.
20. Gartel AL: Mechanisms of apoptosis induced by anticancer compounds in melanoma cells. *Curr Top Med Chem* 12: 50-52, 2012.
21. Chen Z, Xiao Z, Gu WZ, Xue J, Bui MH, Kovar P, Li G, Wang G, Tao ZF, Tong Y, *et al*: Selective Chk1 inhibitors differentially sensitize p53-deficient cancer cells to cancer therapeutics. *Int J Cancer* 119: 2784-2794, 2006.
22. Hirose Y, Berger MS and Pieper RO: Abrogation of the Chk1-mediated G(2) checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer Res* 61: 5843-5849, 2001.
23. Kawabe T: G2 checkpoint abrogators as anticancer drugs. *Mol Cancer Ther* 3: 513-519, 2004.
24. Karunakaran D, Rashmi R and Kumar TR: Induction of apoptosis by curcumin and its implications for cancer therapy. *Curr Cancer Drug Targets* 5: 117-129, 2005.
25. Wang WZ, Cheng J, Luo J and Zhuang SM: Abrogation of G2/M arrest sensitizes curcumin-resistant hepatoma cells to apoptosis. *FEBS Lett* 582: 2689-2695, 2008.
26. Sridhar SS, Seymour L and Shepherd FA: Inhibitors of epidermal-growth-factor receptors: A review of clinical research with a focus on non-small-cell lung cancer. *Lancet Oncol* 4: 397-406, 2003.
27. Okita R, Wolf D, Yasuda K, Maeda A, Yukawa T, Saisho S, Shimizu K, Yamaguchi Y, Oka M, Nakayama E, *et al*: Contrasting effects of the cytotoxic anticancer drug gemcitabine and the EGFR tyrosine kinase inhibitor gefitinib on NK cell-mediated cytotoxicity via regulation of NKG2D ligand in non-small-cell lung cancer cells. *PLoS One* 10: e0139809, 2015.
28. Cui F, Wang W, Wu D, He X, Wu J and Wang M: Overexpression of cathepsin L is associated with gefitinib resistance in non-small cell lung cancer. *Clin Transl Oncol* 18: 722-727, 2016.
29. Li N, Li H, Su F, Li J, Ma X and Gong P: Relationship between epidermal growth factor receptor (EGFR) mutation and serum cyclooxygenase-2 level, and the synergistic effect of celecoxib and gefitinib on EGFR expression in non-small cell lung cancer cells. *Int J Clin Exp Pathol* 8: 9010-9020, 2015.