Original Article

The effects of Benjakul extract and its isolated compounds on cell cycle arrest and apoptosis in human non-small cell lung cancer cell line NCI-H226

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Abstract

Background and purpose: Benjakul, a traditional Thai formulation for cancer treatment, is composed of five plants. This study aimed to assess the cytotoxicity of Benjakul, its five plants, and its isolated compounds against non-small cell lung cancer (NSCLC) by the sulforhodamine B (SRB) assay.
Experimental approach: Analyses of cell cycle and membrane asymmetry changes were performed with different fluorescent dyes and analyzed by flow cytometry in NCI-H226 cells. Activation of caspase-3 was measured using a caspase-3 colorimetric assay kit. The pan-caspase inhibitor Z-VAD-FMK was used in analyses of cell cycle and caspase-3 activity.
Findings/Results: Benjakul exhibited cytotoxicity against NSCLC with IC_{50} between 5.56-5.64 μg/mL. Among its five ingredients, Benjakul displayed the highest selectivity with selectivity index values ranging from 2.93 to 6.88, with the exception of *Plumbago indica*, indicating its protective effects. Plumbagin and 6-shogaol displayed the highest cytotoxicity and underwent molecular studies in NCI-H226 cells. Flow cytometry analysis revealed that Benjakul and 6-shogaol dose-dependently induced G2/M phase arrest, and plumbagin dose-dependently induced S-G2/M phase arrest with the highest percentage in early incubation time (12-24 h). At the highest doses, Benjakul extract, 6-shogaol, and plumbagin time-dependently increased the population of sub-G1 apoptotic cells with the highest percentage in longer incubation time (60-72 h). Similarly, membrane asymmetry changes showed time-dependent increases in the percentage of early and late apoptotic cells. Moreover, the apoptosis-inducing effect of Benjakul, 6-shogaol, and plumbagin at the highest dose, via the caspase cascade was confirmed by time-dependent induction of caspase-3 activity, followed by its complete reduction and abolished sub-G1 peaks upon addition of Z-VAD-FMK.
Conclusion and implication: Our findings demonstrated for the first time the effects of Benjakul and its compounds on S-G2/M or G2/M phase arrest and caspase-dependent apoptosis in lung cancer cells.

Keywords: Apoptosis; Benjakul; Cytotoxic activity; Plumbagin; 6-Shogaol.

INTRODUCTION

Lung cancer is a leading cause of death worldwide (1). The use of complementary and alternative medicine for cancer treatment, along with standard medical treatments, has grown rapidly over the last two decades (2).

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Benjakul, a traditional Thai formulation, is composed of five plants in equal amounts: fruits of *Piper chaba* Hunt. (Piperaceae), roots of *Piper sarmentosum* Roxb. (Piperaceae), stems of *Piper interruptum* Opiz. (Piperaceae), roots of *Plumbago indica* Linn. (Plumbaginaceae) and rhizomes of *Zingiber officinale* Roscoe. (Zingiberaceae). This formulation has long been used to treat cancer patients, and Thai folk doctors cancer patients after its treatment have suggested an increased survival rate although this requires further well-designed clinical trials. Our previous studies have shown that Benjakul and its isolated compounds exerted a cytotoxic effect against breast adenocarcinoma cells (3), large cell lung cancer cell lines (4), and small cell lung cancer cells (5). The active compounds of Benjakul against small lung cancer were plumbagin and 6-shogaol (5). Over the years, plant extracts and plant-derived compounds have been shown to exhibit anticancer activity by activating the apoptotic pathway (6-9). In particular, some isolated compounds from individual plants in the Benjakul formulation exerted cytotoxic effects via apoptotic signaling (10-13). These collective data *suggest* that the apoptosis pathway is likely to be a major mechanism underlying the cytotoxic effect of Benjakul against cancer cells. Therefore, the present study demonstrated the effects of Benjakul and its isolated compounds on cell cycle arrest and apoptosis in NCI-H226 cells, these results support the current use of Benjakul as a complementary and alternative medicine for the treatment of lung cancer.

MATERIALS AND METHODS

Reagents and materials

Authentic compounds, namely plumbagin and 6-shogaol, were purchased from Sigma-Aldrich (St. Louis, *Missouri,* USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Trypsin-EDTA, fetal bovine serum (FBS), antibiotics penicillin and streptomycin, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, and phosphate-buffered saline (PBS) were purchased from Gibco®, Life Technologies (New York, USA). Dimethylsulfoxide (DMSO), tris base, and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, *Missouri,* USA). propidium iodide (PI)/RNase staining solution and annexin V FITC apoptosis detection kit was purchased from BD Pharmingen™ (Bangkok, Thailand). CaspACE™ Assay System colorimetric kit was purchased from Promega (Bangkok, Thailand).

Preparation of extracts and pure compounds

Five plants, comprising Benjakul herbal formulation: the fruits of *Piper chaba* Hunt. (Piperaceae), the roots of *Piper sarmentosum* Roxb. (Piperaceae), the stems of *Piper interruptum* Opiz. (Piperaceae), the roots of *Plumbago indica* Linn. (Plumbaginaceae), and the rhizomes of *Zingiber officinale* Roscoe. (Zingiberaceae) were collected from Chantaburi Province, Thailand. Authentication of plant materials was performed by comparing them against the specimens deposited at the herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand, where the herbarium vouchers were kept (Herbarium no. SKP 146160301, SKP 146161901, SKP 146160901, SKP 148160901 and SKP 206261501, respectively). As previously described, Benjakul and its five plant ingredients were macerated with 95% ethanol to obtain crude extracts. Bioassay-guided fractionation was used to isolate pure compounds from Benjakul extract. Results revealed that the chloroform fraction showed the highest activity against all of the non-small cell lung cancers (NSCLC). Eight compounds were isolated from the chloroform fraction, *e.g*. piperine (BJK-P1), plumbagin (BJK-P2), 6-gingerol (BJK-P3), myristicin (BJK-P4), methyl piperate (BJK-P5), β-sitostenone (BJK-P6), β-sitosterol-stigmasterol (BJK-P7), and 6-shogaol (BJK-P8). The structures of all pure compounds were confirmed by comparing them with previously reported 1H- and 13C-NMR spectral data (5). The crude extracts and all isolated pure compounds were dissolved in DMSO before being tested.

Cell culture

Three types of human lung cancer cell lines of different histological backgrounds (NSCLC) were used. COR-L23 (ECACC 92031919) was purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK). A549 (ATCC® CCL-185™) and NCI-H226 (ATCC® CRL-5826™) were purchased from American Type Culture Collection (VA, USA), and MRC-5 (a normal human lung fibroblast cell line) was purchased from Cell line service GmbH (Eppelheim, Germany). COR-L23, A549, and NCI-H226 were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 IU/mL penicillin, and 50 µg/mL streptomycin. MRC-5 was cultured in DMEM culture medium supplemented with 10% FBS, 50 IU/mL penicillin and 50 µg/mL streptomycin. The cells were maintained at 37 °C in an incubator with 5% CO_2 and 95% humidity.

Cytotoxicity assessment

Screening of cytotoxic effect of five individual plants, Benjakul, and its isolated compounds was carried out on COR-L23, A549, NCI-H226, and MRC-5. The cytotoxic effect was determined by the SRB assay (14). Briefly, cells were seeded in 96-well plates overnight (COR-L23 and A549 = 1×10^3 cells/well, NCI-H226 = 3×10^3 cells/well, and MRC-5 = 5×10^3 cells/well). After 72 h of incubation with various concentrations of the extracts and pure compounds, the cells were rinsed with PBS and incubated with fresh medium at 37 °C for a recovery period. After 72 h of incubation, cells were fixed with cold 40% trichloroacetic acid and incubated at 4 °C for 1 h. Plates were rinsed five times with cold water, then stained with 0.4% SRB solution for 30 min, and the unbound dye was washed five times with 1% acetic acid. SRB dye within the cells was dissolved by adding 10 mM tris base solution. The optical density of the extracted SRB dye was measured with a power wave X plate reader at 492 nm. The 50% inhibitory concentration (IC_{50}) values were calculated using the GraphPad Prism program. In all experiments, paclitaxel was used as a positive control (15). The results are expressed as the average of triplicate assays.

Cell cycle analysis

To estimate the proportion of cells in different phases of the cell cycle, cellular DNA was analyzed by a flow cytometer (Becton-Dickinson FACScan, BD Biosciences, San Jose, USA). Briefly, NCI-H226 cells were seeded in 6-well plates overnight. After various concentrations and times of incubation with Benjakul extract and its isolated compounds (6-shogaol and plumbagin), the cells were harvested, pelleted by centrifugation, and fixed overnight in 70% ice-cold ethanol at -20 °C. The cells were washed with PBS and stained with PI/RNase staining solution for 15 min at room temperature in the dark. These stained cells were collected using a flow cytometer and analyzed for cell cycle phases with ModFit LT 3.2 Software (Verity Software house, USA). The sub-G_1 population was calculated to estimate the apoptotic cell population.

Annexin V/PI staining assay

NCI-H226 cells were treated according to the cell cycle analysis. After a period of exposure, both floating and attached cells were collected by trypsinization, washed with PBS, and pelleted by centrifugation. The cells were stained using the annexin V FITC apoptosis detection kit. The cell pellets were re-suspended in 100 µL of 10% annexin V binding buffer, then 5 µL of annexin V labeling reagent and 5 µL of PI were added and incubated for 15 min in the dark place at room temperature. These cells were then collected using a flow cytometer and analyzed with BD CellQuest™ Software package for Macintosh.

Apoptotic sub G1 population in the presence of pan-caspase inhibitor Z-VAD-FMK

NCI-H226 cells were pretreated with each concentration of the pan-caspase inhibitor Z-VAD-FMK ranging 0.195-50 µM (final DMSO concentrations in a range of 0.5-1%) for 24 h prior to the addition of 150 µg/mL Benjakul extract, 10 µg/mL 6-shogaol, or 1.5 µg/mL plumbagin. After the selected exposure time of each sample, cells were harvested, pelleted by centrifugation, and fixed overnight in 70% ice-cold ethanol at -20 °C. The cells were stained with PI/RNase, and % cell populations in different phases of the cell cycle were measured using the same method as cell cycle analysis.

Caspase-3 activity assay

Caspase-3 activity was determined by CaspACE™ assay system, colorimetric kit. NCI-H226 cells were either un-pretreated or pretreated with 12.5 μmol Z-VAD-FMK prior to the addition of 150 μg/mL Benjakul extract, 10 μg/mL 6-shogaol, or 1.5 μg/mL plumbagin. After a period of exposure, the cell pellets were suspended in 50 μL of 1X lysis buffer and incubated on ice for 15 min. Then the lysed cells were centrifuged, and the supernatants were transferred to new tubes. The protein concentration of each sample was determined using the Bradford protein assay kit (Bio-Rad Laboratories). Each sample which is equal to 50-100 μg range of total protein was transferred to a new microtube, and 35 μL of caspase assay buffer, 2 μL of DMSO, 10 μL of 100 mM dithiothreitol, and deionized water were added to the final volume of 98 μL. The reaction was started by adding 2 μL of caspase substrate to each tube, mixed gently, then transferred to 96-well plates, and incubated for 4 h at 37 °C. The produced chromophore p-nitroanilide was measured at 405 nm. The changes in caspase-3 activity treated with 150 μg/mL Benjakul extract, 10 μg/mL 6-shogaol, or 1.5 μg/mL plumbagin in the presence or absence of Z-VAD-FMK relative to the control were quantified.

Statistical analysis

Results are expressed as mean ± SD of at least three separate experiments. Statistical differences between treatment groups were analyzed by a one-way analysis of variance (ANOVA) followed by post-hoc analysis using Bonferonni Procedure. A P value < 0.05 was considered statistically significant. Statistical differences between the control and treated groups were analyzed using an independent-samples t-test.

RESULTS

Cytotoxic effects of the extracts of Benjakul, its five plant ingredients, and isolated compounds

The cytotoxic activity of Benjakul and its five plants was expressed as an IC_{50} value (Table 1). Also, IC_{50} values of its isolated compounds including myristicin, plumbagin, methyl piperate, 6-shogaol, 6-gingerol, and piperine are shown in Table 2. Paclitaxel, a chemotherapy medication, was used as a positive control. The results demonstrated that the ethanolic extract of Benjakul, its five plants, and its isolated pure compounds exerted cytotoxicity of varying degrees against three types of NSCLC cells. Remarkably, the ethanolic extract of Benjakul exhibited higher cytotoxicity against these cells than almost all of its individual plant extracts and of its isolated compounds. More importantly, Benjakul displayed the highest selectivity relative to MRC-5 normal lung fibroblasts, except for *Plumbago indica*, indicating that Benjakul showed synergistic protective effects against side effects of its individual plants. Among the isolated compounds, plumbagin and 6-shogaol displayed the highest cytotoxic effect.

Table 1. Percentage yields and IC_{50} values of Benjakul extract and its plant ingredients against three human lung cancer cell lines A549, NCI-H226, and COR-L23 versus the human lung fibroblast cell line MRC-5.

Plants	Yield (% w/w)	IC_{50} values in different cell lines			
		A549	NCI-H226	COR-L23	MRC-5
Piper chaba	10.89	23.83 ± 0.12[a] 3.85[b]	35.40 ± 0.28[a] 2.59[b]	33.55 ± 0.31[a] 2.73[b]	91.71 ± 0.50[a]
Piper sarmentosum	6.21	70.80 ± 0.17[a] > 1.41[b]	64.39 ± 0.20[a] > 1.55[b]	45.99 ± 0.85[a] > 2.17[b]	> 100[a]
Piper interruptum	2.47	48.10 ± 1.60[a] 0.72[b]	44.01 ± 0.44[a] 0.78[b]	48.55 ± 0.16[a] 0.71[b]	34.44 ± 1.61[a]
Plumbago indica	10.61	5.98 ± 0.93[a] 11.71[b]	5.64 ± 0.06[a] 12.42[b]	5.56 ± 0.20[a] 12.59[b]	70.04 ± 2.16[a]
Zingiber officinale	4.30	54.66 ± 0.06[a] 1.53[b]	55.60 ± 0.05[a] 1.50[b]	55.58 ± 0.56[a] 1.50[b]	83.45 ± 5.37[a]
Benjakul	9.11	10.18 ± 0.73[a] 6.88[b]	13.00 ± 0.72[a] 5.39[b]	23.91 ± 3.27[a] 2.93[b]	70.07 ± 2.46[a]

[a] The IC_{50} value in μg/mL is expressed as the mean ± SEM of 4 replicates from three separate experiments; [b] the selectivity index = IC_{50} of the extract in MRC-5 / IC_{50} of the extract in the cancer cell line.

Table 2. IC_{50} values of active compounds isolated from Benjakul extract against three human lung cancer cell lines A549, NCI-H226, and COR-L23 versus the human lung fibroblast cell line MRC-5.

Compounds	IC_{50} values in different cell lines			
	A549	NCI-H226	COR-L23	MRC-5
BJK-P1 (Piperine)	79.82 ± 1.55[a] 279.74[b] 0.49[c]	23.89 ± 0.54[a] 83.72[b] 1.63[c]	21.33± 0.66[a] 74.75[b] 1.82[c]	38.83 ± 4.97[a] 136.08[b]
BJK-P2 (Plumbagin)	0.53 ± 0.01[a] 2.82[b] 1.83[c]	0.57 ± 0.16[a] 3.03[b] 1.72[c]	0.47 ± 0.11[a] 2.50[b] 2.07[c]	0.97 ± 0.58[a] 5.15[b]
BJK-P3 (6-gingerol)	61.27 ± 0.51[a] 208.13[b] 1.46[c]	42.60 ± 2.80[a] 144.71[b] 2.10[c]	58.46 ± 4.65[a] 198.58[b] 1.53[c]	89.33 ± 10.50[a] 303.44[b]
BJK-P4 (Myristicin)	69.13 ± 0.26[a] 359.19[b] 1.17[c]	> 100[a] > 520.26[b]	> 100[a] > 520.26[b]	80.69 ± 5.40[a] 419.80[b]
BJK-P5 (Methyl piperate)	37.60 ± 1.13[a] 161.91[b] 1.54[c]	23.02 ± 0.51[a] 99.11[b] 2.52[c]	22.20 ± 5.38[a] 95.59[b] 2.61[c]	57.99 ± 10.38[a] 249.69[b]
BJK-P6 (6-shogaol)	5.15 ± 0.15[a] 18.63[b] 0.99[c]	4.98 ± 0.03[a] 18.02[b] 1.03[c]	4.48 ± 0.42[a] 16.21[b] 1.14[c]	5.12 ± 0.50[a] 18.53[b]
Paclitaxel	0.0021 ± 0.00004[a] 0.0025[b] 9.40[c]	0.0037 ± 0.00002[a] 0.0043[b] 5.41[c]	0.0018 ± 0.00023[a] 0.0021[b] 11.03[c]	0.0120 ± 0.011[a] 0.0141[b]

[a] The IC_{50} value in µg/mL is expressed as the mean ± SEM of 4 replicates from three separate experiments; [b] The mean IC_{50} value is also expressed in µM; [c] the selectivity index (IC_{50} of the compound in MRC-5 / IC_{50} of the compound in cancer cell line).

Inhibitory effects of Benjakul extract, 6-shogaol, and plumbagin on cell cycle progression

Because Benjakul extract and its isolated compounds (6-shogaol and plumbagin) also showed *prominent* cytotoxicity against squamous NCI-H226 cells, these cells were further chosen to determine the inhibitory effect on cell cycle progression and the presence of the sub-G_1 population (apoptotic cells). Flow cytometry analysis showed that Benjakul extract and 6-shogaol dose-dependently arrested NCI-H226 cells at the G2/M phase with the highest percentage at 12 and 24 h of incubation, respectively (Fig. 1). With minor differences, plumbagin dose-dependently induced S-G2/M phase arrest with the highest percentage at 12 h of incubation (Fig. 1). At the highest concentrations used, 150 µg/mL Benjakul extract, 10 µg/mL 6-shogaol, and 1.5 µg/mL plumbagin time-dependently increased the population of sub-G1 apoptotic cells with the highest percentage of these cells at 60, 72, 60 h of incubation, respectively, with parallel decreases in arrested cell cycle phases

(Fig. 2). Taken together, these results indicated that Benjakul extract, 6-shogaol, and plumbagin caused cell cycle arrest, ultimately leading to apoptosis.

Induction of early apoptosis by Benjakul extract, 6-shogaol, and plumbagin

As the highest concentration of Benjakul extract, 6-shogaol, and plumbagin time-dependently increased the population of sub-G1 apoptotic cells with parallel decreases in arrested cell cycle phases (Fig. 2), describing that cell cycle arrest preceded apoptosis. Therefore, the apoptotic effect of Benjakul extract, 6-shogaol, and plumbagin was further analyzed by staining the cells with annexin V FITC and PI for the measurement of early apoptotic cells using flow cytometry. In this particular, 150 µg/mL Benjakul extract induced *approximately* 60% of combined early and late apoptotic cells at 60 h of incubation. Similarly, 10 µg/mL 6-shogaol and 1.5 µg/mL plumbagin caused about 80% of combined early and late apoptotic cells at 72 and 60 h of incubation, respectively. It was apparent that, in

each treatment, the highest *percentage of* combined apoptotic cells (Fig. 3) corresponded to the highest percentage of the sub-G_1 peak in the cell cycle analysis (Fig. 2) *in* a time-dependent manner. This finding indicated that Benjakul extract and its isolated compounds induced cytotoxicity in NCI-H226 cells through the induction of apoptosis.
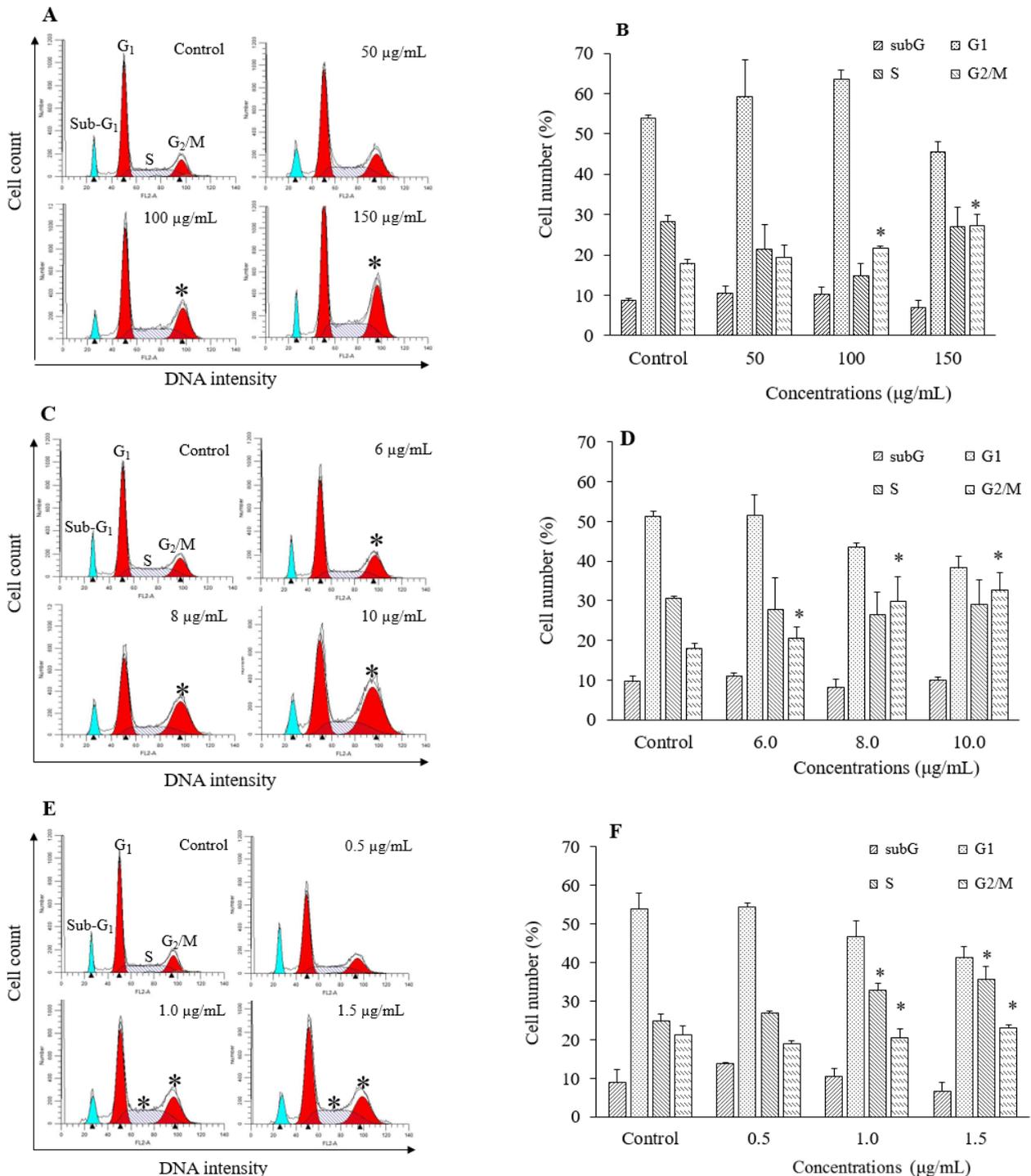

Fig. 1. Concentration-dependent effects of Benjakul, 6-shogaol, and plumbagin on cell cycle of NCI-H226 cell. Cells were incubated in the absence (control) or with Benjakul for 12 h, 6-shogaol for 24 h, or plumbagin for 12 h, and analyzed by flow cytometry and ModFit LT® software. Histogram plots and bar graphs showing the percentage of cells in the cell cycle phase from cells treated with (A and B) Benjakul, (C and D) 6-shogaol, or (E and F) plumbagin. Values represent means ± SD of three independent experiments. *$P < 0.05$ indicates significant differences compared with the control.
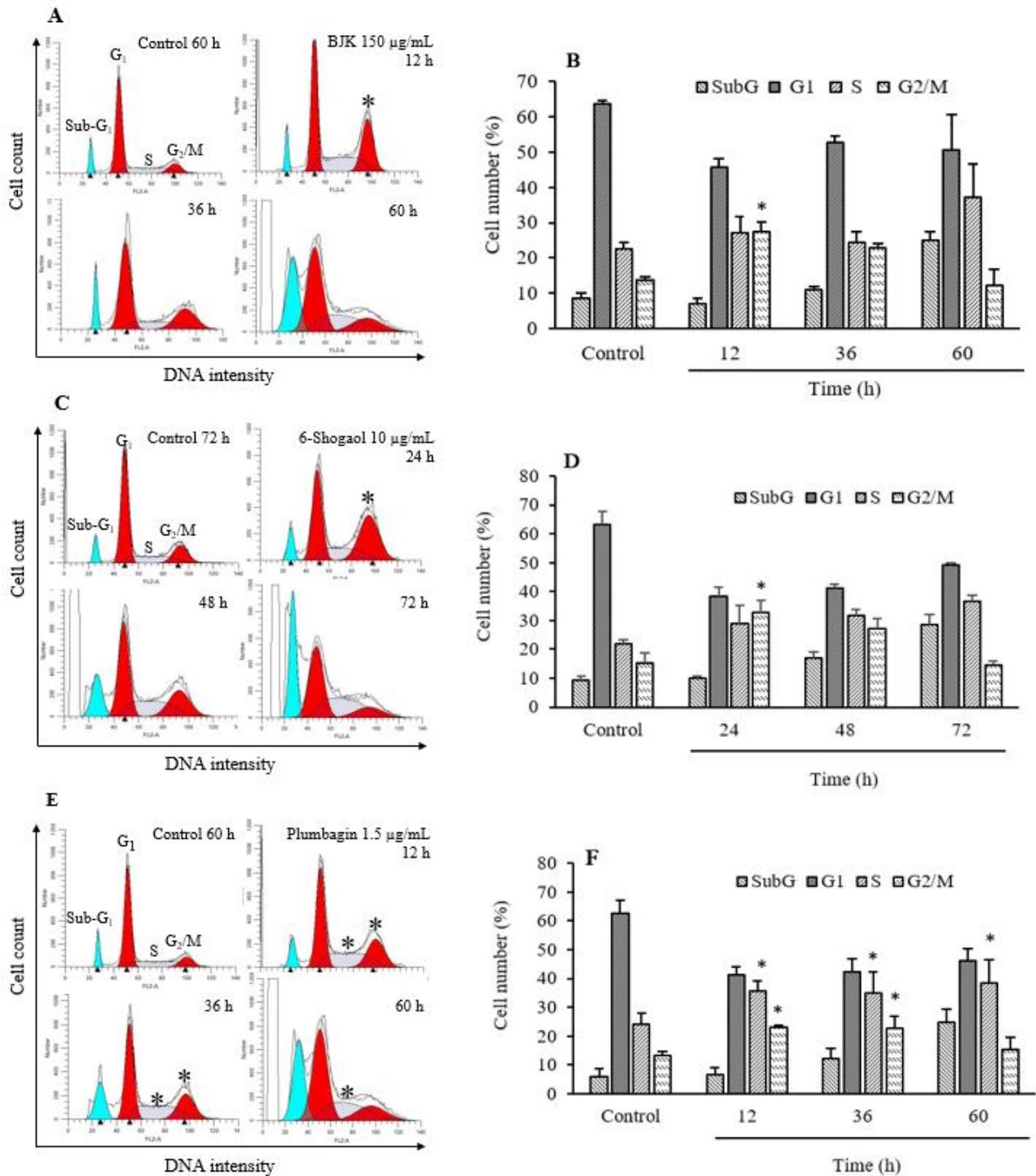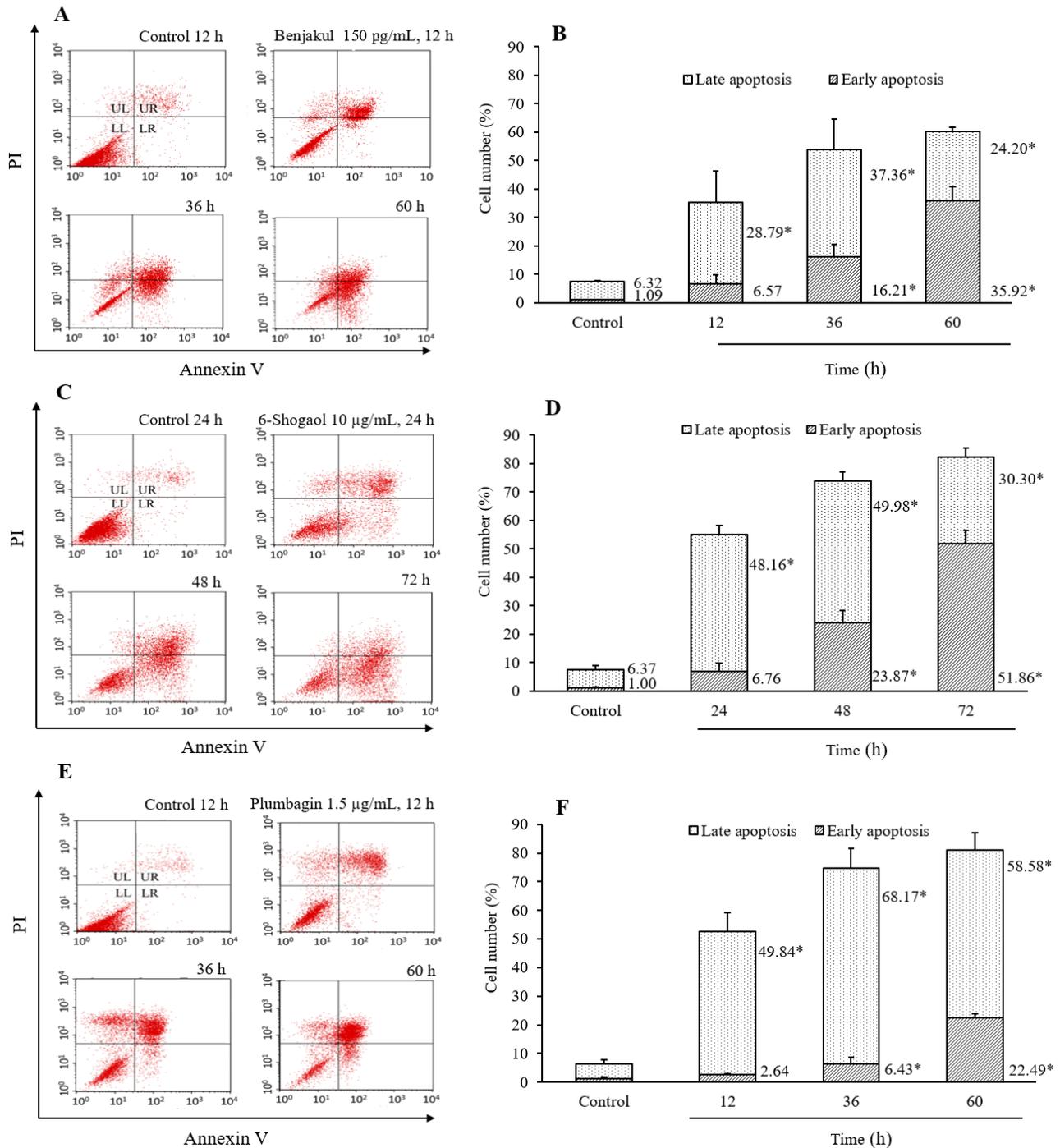
Fig. 2. Time-dependent effects of Benjakul, 6-shogaol, and plumbagin on cell cycle of NCI-H226 cell. Cells were incubated in the absence (control), or Benjakul at 150 μg/mL, 6-shogaol at 10.0 μg/mL, or plumbagin at 1.5 μg/mL, and then analyzed by flow cytometry and ModFit LT® software. Histogram plots and bar graphs showing the percentage of cells in the cell cycle phase from cells treated with (A and B) Benjakul, (C and D) 6-shogaol, or (E and F) plumbagin. Values represent means ± SD of three independent experiments. *$P < 0.05$ indicates significant differences compared with the control.

Fig. 3. Time-dependent effects of Benjakul, 6-shogaol, and plumbagin on early apoptosis induction in NCI-H226 cell. Cells were incubated in Benjakul at 150 μg/mL for, 6-shogaol at 10.0 μg/mL, or plumbagin at 1.5 μg/mL, and analyzed by flow cytometry. Dot plots and bar graphs showing the percentage of early apoptotic (lower right, LR) and late apoptotic cells (upper right, UR) from cells treated with (A and B) Benjakul, (C and D) 6-shogaol, or (E and F) plumbagin. Values represent means ± SD of three independent experiments. *$P < 0.05$ indicates significant differences compared with the control.

Suppression of apoptosis induced by Benjakul extract, 6-shogaol, and plumbagin with the use of the general caspase inhibitor Z-VAD-FMK

Sub-G$_1$ peaks and early apoptotic cells indicated the involvement of the apoptotic signaling pathway. Therefore, we next investigated whether NCI-H226 cells treated with Benjakul extract, 6-shogaol, and plumbagin underwent apoptosis *via* caspase cascade by measuring cells in the sub-G$_1$ phase of the cell cycle in the absence or presence of Z-VAD-FMK, a cell-permeable pan-*caspase inhibitor*. As shown in Fig. 4, pretreatment of NCI-H226 cells with Z-VAD-FMK ranging 0.195-50 μM before the addition of 150 μg/mL Benjakul extract, 10 μg/mL 6-shogaol, or 1.5 μg/mL plumbagin,

concentrations-dependently decreased sub-G_1 peaks with significant differences. *The results demonstrated that* Z-VAD-FMK *at concentrations with an asterisk in Fig. 4 significantly blocked apoptosis induced by Benjakul extract, 6-shogaol, and plumbagin. The highest concentration of Z-VAD-FMK at 50 μM decreased sub-G_1 peak to the* same level *as that of the* control, *thus suggesting that Benjakul extract and its compounds induced apoptosis through the caspase-dependent pathway.*

Effect of Benjakul extract, 6-shogaol, and plumbagin on caspase-3 activation

As caspase-3 plays a key factor in apoptosis execution, we next determined whether caspase-3 activity in NCI-H226 cells was induced by the treatment of the highest concentration of Benjakul extract, 6-shogaol, or plumbagin. The result showed that a 3- to 4-fold activation of caspase-3 was detected in all treated cells relative to untreated cells at different exposure times (Fig. 5). Activation of caspase-3 was confirmed by the use of the pan-caspase inhibitor Z-VAD-FMK. Interestingly, the highest caspase-3 activity in all treated cells was completely inhibited in the presence of 12.5 μM Z-VAD-FMK (Fig. 5). Therefore, these results demonstrated that the ethanolic extract of Benjakul, 6-shogaol, and plumbagin induced apoptosis in NCI-H226 cells *via* the caspase-dependent pathway where the caspase-3 is a key executor.
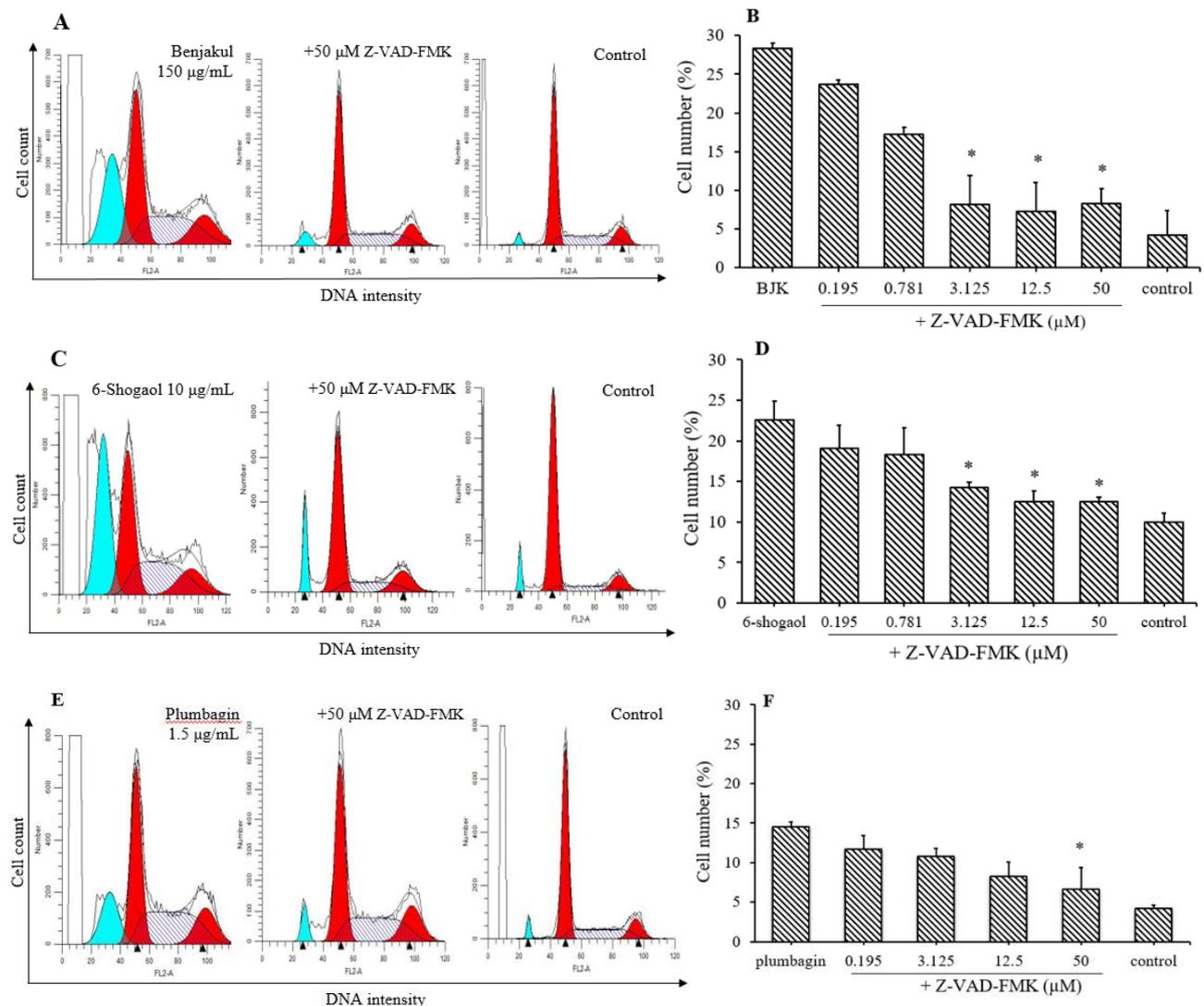

Fig. 4. Inhibitory effects of Z-VAD-FMK on sub-G1 peak in NCI-H226 cells treated with Benjakul, 6-shogaol, and plumbagin. Cells were pretreated with Z-VAD-FMK at 0.195, 0.78, 3.125, 12.5, and 50 μM or without Z-VAD-FMK (control) for 24 h before the addition of 150 μg/mL Benjakul for 60 h, 10 μg/mL 6-shogaol for 72 h, or 1.5 μg/mL plumbagin for 60 h. Histogram and bar graphs showed the percentage of sub-G1 cells in Z-VAD-FMK-pretreated cells before the addition of (A and B) Benjakul, (C and D) 6-shogaol, or (E and F) plumbagin. Data are expressed as mean ± SD of three independent experiments. *$P < 0.05$ indicates significant differences compared with the control.
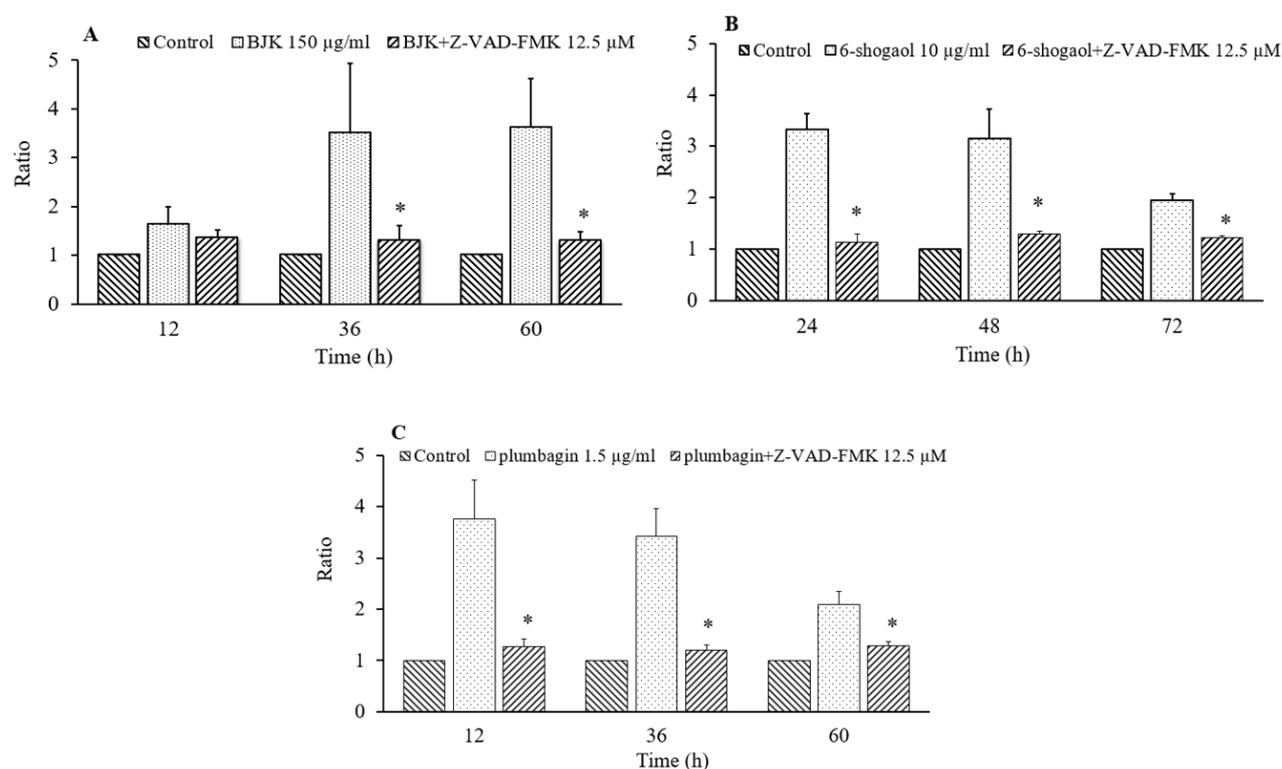
Fig. 5. Inhibitory effects of Z-VAD-FMK on caspase-3 activity in NCI-H226 cells treated with Benjakul, 6-shogaol, and plumbagin. Cells were pretreated with 12.5 μM Z-VAD-FMK or without Z-VAD-FMK (control) for 24 h before addition of 150 μg/mL Benjakul for 12, 36, 60 h, 10 μg/mL 6-shogaol for 24, 48, 72h, or 1.5 μg/mL plumbagin for 12, 36, 60 h. Bar graphs representing fold changes in caspase-3 activity in cells pretreated with or without Z-VAD-FMK before the addition of (A) Benjakul, (B) 6-shogaol, or (C) plumbagin. Data are expressed as mean ± SD of three independent experiments. *$P < 0.05$ indicates significant differences compared with the control.

DISCUSSION

Benjakul extract exhibited stronger cytotoxicity against all three types of NSCLC than almost all of its individual plants and of its isolated compounds. With the exception of *P. indica*, Benjakul exerted the highest cytotoxic selectivity, indicating considerable loss of such selectivity in these plant extracts and compounds. Although *Piper indica* exhibited higher degrees of cytotoxic selectivity against all NSCLC, it cannot be used independently clinically due to its adverse effects, such as severe skin irritation. According to the Food and Drug Administration (*FDA)* in Thailand, individual plants with these effects are allowed to be used only as ingredients with limited amounts in Thai herbal remedies. Our data suggest synergistic protective effects of Benjakul remedy against side effects of its individual plants and provides underlying scientific data consistent with its traditional use. This remedy has long been used in Thailand, with no anecdotal data on any side effects.

Similarly, to some extent, synergistic anti-inflammatory effects of Benjakul over those of its individual plants have been possible. Among isolated compounds of Benjakul extract, plumbagin, and 6-shogaol displayed the highest cytotoxic effect and may have potential as its bioactive *markers*.

Currently, cell viability and cytotoxicity assays with cultured cells are widely used to assess cellular or metabolic changes associated with viable or nonviable cells. Each assay has its own set of advantages and limitations. Some of these assays can detect physiological and biochemical activities indicative of living cells, which are based on various cell functions such as enzyme activity, ATP production, and so forth. For example, the MTT assay determines cell viability through the determination of mitochondrial functions of cells by measuring the activity of mitochondria enzymes. Despite its easy use, safe, and high reproducibility, recent studies have shown that MTT can be reduced in the absence of living cells by several phenolics, e.g., stilbenes and flavonoids, e.g.,

quercetin, which is commonly found in plants, possibly generating artifacts. In addition, cytotoxicity assays, such as dye exclusion assays, measure the loss of membrane integrity upon cell death, but a large number of samples is difficult and time-consuming. The lactate dehydrogenase (LDH) assay detects the release of this cytosolic enzyme from damaged cells due to their lost membrane integrity. The major limitation of this assay is that serum and some other compounds have inherent LDH activity. The SRB assay is our selection for measuring cytotoxicity in which this aminoxanthene dye binds to protein basic amino acid residues in trichloroacetic acid-fixed cells to provide a sensitive index of cellular proteins. As we did not face the limitations of this assay, which are the avoidance of cell clumps or aggregates, the SRB assay is thus most appropriate for our study.

Based on the effects on cell cycle progression, Benjakul extract, plumbagin, and 6-shogaol acted in the same direction through triggering cell cycle arrest in a time- and concentration-dependent manner. Moreover, our data demonstrated the apoptotic effect of Benjakul extract and its isolated compounds, to a certain extent, *via* sub-G1 peaks (representing apoptotic cells). By using annexin V/PI staining and flow cytometry, early apoptotic cells with intact membranes (annexin V + / PI-; an indicator of apoptotic induction) and late apoptotic cells with leaky membranes (annexin V + / PI+) were also revealed in a time-dependent manner. This apoptosis-inducing effect *via* the caspase cascade was also demonstrated with a 3- to 4-fold activation of caspase-3 activity. Upon addition of Z-VAD-FMK, a cell-permeable pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases (17,18), such increases of caspase-3 activity and sub-G1 peaks were almost completely abrogated. In particular, this inhibitor could not prevent the caspase-independent apoptosis pathway (19). These collective data suggested the potential use of plumbagin and 6-shogaol as biological representative markers for Benjakul preparation. Despite different types of NSCLC cells, the effects of plumbagin and 6-shogaol isolated from Benjakul remedy on G_2/M arrest and caspase-dependent apoptosis were consistent with the pure compounds in the previous studies reporting their effects *via* the function of p53, a critical tumor suppressor (11,12). On the contrary to these studies, however, squamous NCI-H226 cells used in our study have been reported to contain mutation sites in p53 (20); hence, the effects of Benjakul extract, plumbagin, and 6-shogaol on cell cycle arrest and caspase-dependent apoptosis were likely to be attributed to p53 independent-alternative pathways. However, additional assessments of apoptosis markers (BAX, BAD, BCL-2 protein levels) are needed to truly confirm the involvement of the apoptotic signaling pathways.

CONCLUSION

These data demonstrated that Benjakul extract, plumbagin, and 6-shogaol induced G_2/M cell cycle arrest and caspase-dependent apoptosis. The results appropriately support the traditional use of Benjakul and the further investigation of its extract and components in the treatment of cancer. However, larger human studies would be needed to recommend it as a safe and effective treatment in general.

Conflict of interest statement
The authors declared no conflict of interest in this study.

Authors' contributions
A. Itharat and P. Hansakul contributed to the design of all experiments; A. Itharat, R. Rattarom, and I. Sakpakdeejaroen participated in extracting and isolating pure compounds from Benjakul; A. Itharat and R. Rattarom participated in the cytotoxic experiment; A. Itharat, R. Rattarom, and P. Hansakul participated in apoptotic experiments and interpretation of these parts; A. Itharat, P. Hansakul, R. Rattarom, and

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