

Anticancer and Cancer Prevention Effects of Piperine-Free *Piper nigrum* Extract on N-nitrosomethylurea-Induced Mammary Tumorigenesis in Rats

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Abstract

Piper nigrum (*P. nigrum*) is commonly used in traditional medicine. This current study aimed to investigate the anticancer and cancer preventive activity of a piperine-free *P. nigrum* extract (PFPE) against breast cancer cells and N-nitrosomethylurea (NMU)-induced mammary tumorigenesis in rats. The cytotoxic effects and the mechanism of action were investigated in breast cancer cells using the MTT assay and Western blot analysis, respectively. An acute toxicity study was conducted according to the Organization for Economic Co-operation and Development guideline. Female Sprague-Dawley rats with NMU-induced mammary tumors were used in preventive and anticancer studies. The results showed that PFPE inhibited the growth of luminal-like breast cancer cells more so than the basal-like ones by induction of apoptosis. In addition, PFPE exhibited

greater selectivity against breast cancer cells than colorectal cancer, lung cancer, and neuroblastoma cells. In an acute toxicity study, a single oral administration of PFPE at a dose of 5,000 mg/kg body weight resulted in no mortality and morbidity during a 14-day observation period. For the cancer preventive study, the incidence of tumor-bearing rats was 10% to 20% in rats treated with PFPE. For the anticancer activity study, the growth rate of tumors in the presence of PFPE-treated groups was much slower when compared with the control and vehicle groups. The extract itself caused no changes to the biochemical and hematologic parameters when compared with the control and vehicle groups. In conclusion, PFPE had a low toxicity and a potent antitumor effect on mammary tumorigenesis in rats. *Cancer Prev Res*; 9(1); 74–82. ©2015 AACR.

Introduction

One of the most common malignancies in women is breast cancer. It also has a high mortality rate worldwide, with 522,000 deaths in 2012 (1). Therefore, the World Health Organization realizes and promotes breast cancer control that requires multiple actions. These actions are coupled with prevention, early diagnosis, and treatment (2). There are four major procedures for cancer treatment, which include surgery, radiation, hormonal therapy, and chemotherapy. However, the resistance of cancer cells to multiple chemotherapeutic agents and the side effects of some agents pose a problem for the successful treatment of breast cancer. Nowadays, the development of multidrug resistance (MDR) is a major drawback to chemotherapy. Over a long-term

treatment, many patients suffer from MDR, which can reduce therapeutic efficiency and lead to treatment failure and a decrease chance of survival (3, 4). Therefore, the search for new potent chemotherapeutic agents from natural compounds is one way to detect new compounds for cancer treatment. Several medicinal plants have served as anticancer pharmaceutical resources, and over 60% of current anticancer drugs, such as vinblastin, topotecan, etotecan, and paclitaxel are plant-derived compounds (5, 6).

Piper nigrum (*P. nigrum*) or black pepper (Piperaceae) is a traditional medicinal plant used for the treatment of diarrhea, earache, gangrene, cardiovascular diseases, indigestion, and insomnia. *P. nigrum* also can help against respiratory disorders, such as cold, fever, and asthma (7), and has been claimed to have other activities, including anti-inflammatory activity, antithyroid activity, and is chemopreventive (8–9). Several compounds have been isolated from *P. nigrum*, such as alkaloids, lignans, and terpenes (10). Piperine, a major pungent alkaloid, has been reported to have anticancer properties, inhibits lung metastasis induced by B16F-10 melanoma cells in mice (11), and protects against benzo[α]pyrene-induced lung carcinogenesis in Swiss albino mice (12) and in different animal models, such as Balb/c mice and sarcoma 180-transplanted mice (13, 14). In a previous study, we have investigated the cytotoxic effects of the extracts from seven species of plants of genus *Piper*. The result showed that the methanolic and dichloromethane crude extract of *P. nigrum* had a potent cytotoxic effect on the breast cancer cell lines (MCF-7 and MDA-MB-468). According to the results, we decided to focus on a piperine free *P. nigrum* extract (PFPE).

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Moreover, PFPE exhibited higher cytotoxic effects on breast cancer cell lines than piperine (15). However, to date, there has been no study on the anticancer and cancer preventive effects of PFPE. Therefore, this study aimed to evaluate the anticancer and cancer preventive activities of PFPE against breast cancer cell lines and N-nitrosomethylurea (NMU)-induced mammary tumorigenesis in rats.

Materials and Methods

Plant materials

The black peppercorn (*P. nigrum*) was collected from Songkhla province in Thailand. The plant specimen (voucher specimens number SKP 146161401) was identified by Assistant Professor Dr. Supreeya Yuenyongsawad and deposited in the herbarium at the Southern Centre of Thai Traditional Medicine, Department of Pharmacognosy, and Pharmaceutical Botany, Prince of Songkla University, Thailand.

Preparation of plant extracts

PFPE was prepared by Reshmi's method with slight modification (16). The dried fruits of the plant were grounded into small sizes. Two hundred and fifty grams of dried black peppercorn were extracted with 300 mL of dichloromethane for 3 hours at 35°C in a shaker incubator. This solvent-containing extract was filtered using Whatman filter paper (No.1) and concentrated in vacuum below 45°C using a rotary evaporator. The dark brown oily residue was cooled in an ice bath for 5 minutes, and 100 mL of cold diethyl ether was added. The solution was then concentrated by using a rotary evaporator. After cooling in the ice bath for 5 minutes, the oil was dissolved in 100 mL of cold diethyl ether and shaken for 25 minutes. The yellow crystals were filtered using Whatman filter paper (No.3) and discarded. The brown residue was then concentrated in vacuum below 45°C using a rotary evaporator. The concentrated extract was then kept in a desiccator until further use.

Cell lines and culture conditions

Four breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468, and ZR-75-1), a neuroblastoma (SK-N-SH), and a normal breast (MCF-12A) cell line were obtained from the ATCC. Two colorectal carcinoma (HT-29 and SW-620), two lung carcinoma (A-549 and H-358), a neuroblastoma (LA-N-5), and a mouse fibrosarcoma (L-929) cell lines were kindly donated by Associate Professor Dr. Surasak Sangkhatat (Department of Surgery, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand), Associate Professor Dr. Ruedeekorn Wiwattanapatapee (Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand), Associate Professor Dr. Pornprot Limprasert (Department of Pathology, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand), Assistant Professor Dr. Jasadee Kaewsichan (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand), and Dr. Pritsana Raungrut (Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand). The MCF-7, ZR-75-1, A-549, and H-358 cells were grown in RPMI 1640 (Invitrogen) containing 10% FBS (GIBCO BRL) with 2 mmol/L of L-glutamine (Invitrogen) and an antibiotic mixture (Invitrogen) of 100 units/mL of penicillin and 100 µg/mL of streptomycin. MDA-MB-231, MDA-MB-468, HT-

29, SW-620, LA-N-5, SK-N-SH, and L-929 cells were all grown in DMEM (Invitrogen) supplemented with the same supplement as for RPMI 1640. MCF-12A cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium (PAA Laboratories GmbH) containing 5% horse serum (Invitrogen) with 20 ng/mL of human epidermal growth factor (Invitrogen), 100 ng/mL of cholera toxin (Sigma), 0.01 mg/mL bovine insulin (Sigma), and 500 ng/mL of hydrocortisone, 95% (Sigma). All cells were maintained by incubating in a 5% CO₂ atmosphere, at 37 °C, and 96% relative humidity.

In vitro cytotoxicity study (MTT assay)

The *in vitro* cytotoxicity of PFPE on cancer cell lines and two normal cell lines was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. IC₅₀ values were determined and calculated as previously described (15).

The selective index (SI) was used to determine the selectivity of the extracts to the tested cell lines as described by Bézivin and colleagues (17). The SI was determined by the IC₅₀ value of the PFPE on normal cell lines divided by the IC₅₀ value of the PFPE on the cancer cell lines. The higher SI values indicated the more selective extract.

Detection of apoptosis

MCF-7 cells were seeded in 12-well plates at a density of 3×10^4 cells/well for overnight growth before treatment with PFPE. Cells were double stained with the FITC-Annexin V Apoptosis Detection Kit II using the manufacturer's protocol (BD Pharmingen). Apoptotic cells were analyzed on a FACSCalibur [Becton Dickinson Biosciences (BDB)] using Cell Quest software (BDB) equipped with a 488-nm argon ion laser. Five thousand nuclei were obtained with FACS. The data were analyzed using the WinMDI version 2.9 software.

Western blot analysis

MCF-7 cells in their exponential growth phase were seeded into 6 cm culture plates at a density of 4×10^6 cells/plate for 24 hours and then treated with PFPE at its IC₅₀ concentration. After treatment, cells were harvested every day for 4 days and kept at -70°C until used.

Cell pellets were lysed using the RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mmol/L EDTA). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against cytochrome C (cyt C), topoisomerase II (topo II), and GAPDH for 2 hours and p53 for overnight. The membranes were incubated with secondary antibody for 1 hour. Bound antibodies were detected and visualized by a chemiluminescence detection using the SuperSignal West Dura substrate (Pierce).

Animals

Female Imprinting Control Region (ICR) mice and female Sprague-Dawley rats were obtained from the National Laboratory Animal Center, Nakorn Pathom, Thailand. Mice of 8 weeks of age with a weight of 30 to 35 g and rats of 50 days of age with a weight of 150 to 180 g were used. All animals were housed in a temperature-controlled room at 25±1°C with a 12-hour light/dark cycle at the Southern Laboratory Animal facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The animals were fed with food and water *ad libitum*. Before experiments, mice and rats

were acclimatized for 7 days to adapt to the laboratory environment. All procedures were approved by the Ethics Committee on Animal Experiment (Ref. 30/55 and Ref. 19/57), Prince of Songkla University, Thailand.

Acute toxicity study

Acute oral toxicity studies were performed according to the OECD 425 (Organization for Economic Co-operation and Development; ref. 18). Ten adult female ICR mice were divided into two groups. The test group was given PFPE with an oral dose of 5,000 mg/kg body weight once. PFPE was resuspended in the vehicle, a mixture of distilled water and Tween-80 (4:1 v/v). The control group was fed with vehicle. Animals were observed for symptoms of toxicity and mortality, such as convulsion, hyperactivity, sedation, loss of righting reflex, increased or decreased respiration, tremor, salivation, and diarrhea for the first 4 hours and then daily for 14 days.

Cancer preventive activity study

The female Sprague-Dawley rats were divided into four groups, each group consisting of 10 animals. Group I (normal) animals received no treatment and NMU (Sigma Chemical Co.). Animals in Groups II to IV were injected intraperitoneally with 50 mg/kg of NMU to induce breast cancer at 50, 80, and 110 days of age as previously described (19). Group II (control) received a mixture of distilled water and Tween-80 (vehicle). Groups III and IV were orally treated with PFPE at 100 and 200 mg/kg body weight, respectively, at 14 days after the first NMU application. Groups II, III, and IV were treated three times a week for 76 days. The rats were palpated twice weekly for detection of mammary tumors. The developing tumors were measured with a caliper in the two greatest perpendicular dimensions three times a week. The tumor size, body weight, and clinical signs were observed and recorded. The tumor volume was calculated using the equation $V = 0.4ab^2$, where "a" and "b" represent the largest tumor diameter and the next largest tumor diameter as previously described (20). All rats were sacrificed at the end of experiment. The wet tumor and organs, such as lung, liver, kidney, heart, and gastric tissues, were measured and weighed. The ratio of organ weight to the final body weight and their values were expressed as a percentage. For paired organs, the mean weight of the two organs was used to calculate the organs weight/body weight ratio. Blood samples were obtained from rats by cardiac puncture for hematologic and biochemical analysis.

Anticancer activity study

The female Sprague-Dawley rats were divided into six groups, each group consisting of 10 animals. Group I (normal) animals received no treatment or NMU. Group II (negative control) to VI rats were injected with NMU to induce breast cancer at 50, 80, and 110 days of age. In addition, group III (vehicle) received only the vehicle, whereas groups IV, V, and VI were orally treated with 100, 200, and 400 mg/kg body weight of PFPE, respectively. The treatments were started when the first tumors showed a diameter of ≥ 5 mm. All rats received their specific treatment every 2 days until 30 days after the first tumor was detected. The assessment of different treatments was performed using the same procedures as described for the preventive study.

Histopathologic study

The tumor and liver tissues were isolated from the rats at autopsy and fixed with 10% buffered formalin solution. The specimens were washed with 1XPBS, then dehydrated in an ascending series of ethanol, and embedded in a paraffin block. The specimens were sectioned, deparaffinized in xylene, rehydrated with a series of ethanol, and rinsed in distilled water before staining. The sections were then stained with hematoxylin and eosin for histopathologic examination.

Immunohistochemistry

Immunohistochemical examination was performed on a Leica Bond-Max-automated IHC/*In situ* hybridization (ISH) platform (Leica Biosystems Newcastle Ltd). The paraffin-embedded blocks were sectioned and dewaxed in a Bond Dewax solution. The sections were then rehydrated in alcohol and a Bond Wash solution (Leica Microsystems). Antigen retrieval was performed using the retrieval solution for 15 minutes followed by endogenous peroxidase blocking for 5 minutes on the machine. The sections were prepared by immunostaining with anti-ER α (Santa Cruz Biotechnology), anti-HER-2 (Sigma Chemical Co.), and anti-cyt C (Cell signaling Technology, Inc.) antibodies. Detection was performed using the Bond Polymer Refine Red Detection system (Leica Microsystems) with a 15-minute postprimary step followed by 25-minute incubation with the alkaline phosphatase-linked polymers. Sections were then counterstained with hematoxylin on the machine, dehydrated in alcohols, and mounted. The estrogen receptor (ER) immunoreactivity was observed in the nucleus, whereas the human epidermal growth factor receptor (HER-2) was observed in the cell membrane. The cyt C immunoreactivity was observed in the cytoplasm. Strong intensity refers to the darkest brown cytoplasmic staining. The evaluation of the stained tumor cells was carried out by a professional pathologist.

Statistical analysis

The median inhibition concentration (IC₅₀) data were acquired by the SoftMax Pro 5 program (MDS Analytical Technologies Inc.). Animal data were analyzed by one-way ANOVA. All results were represented as a mean value \pm SD. A *P* value of less than 0.05 was considered statistically significant.

Results

Effect of PFPE on the proliferation of breast cancer cell lines

The proliferation of the four breast cancer, two colorectal carcinoma, two lung carcinoma, two neuroblastoma, a normal breast, and mouse fibrosarcoma cell lines was measured using the MTT assay. Previously, we have shown that piperine itself had less effect against breast cancer cell lines than PFPE (15). Therefore, piperine was removed from the crude extract by recrystallization. Here, we confirmed that our recrystallized crude extract had no piperine using LCMS (Fig. 1Aa–Ab). The cytotoxicity of PFPE is shown in Table 1. The PFPE was a highly effective inhibitor against the MCF-7 cells with an IC₅₀ value of 7.45 ± 0.60 μ g/mL. In addition, the SI values of PFPE in the MCF-7 cells were 6.22 and 4.54 when compared with the MCF-12A and L-929, respectively. PFPE had a lower cytotoxic effect on the MCF-12A and L-929 cells. Moreover, PFPE possessed the most selectivity for the breast cancer cell lines and showed lower SI values for other cancer cell lines (colorectal cancer, lung cancer, and neuroblastoma cell lines). Our results indicated that PFPE has a

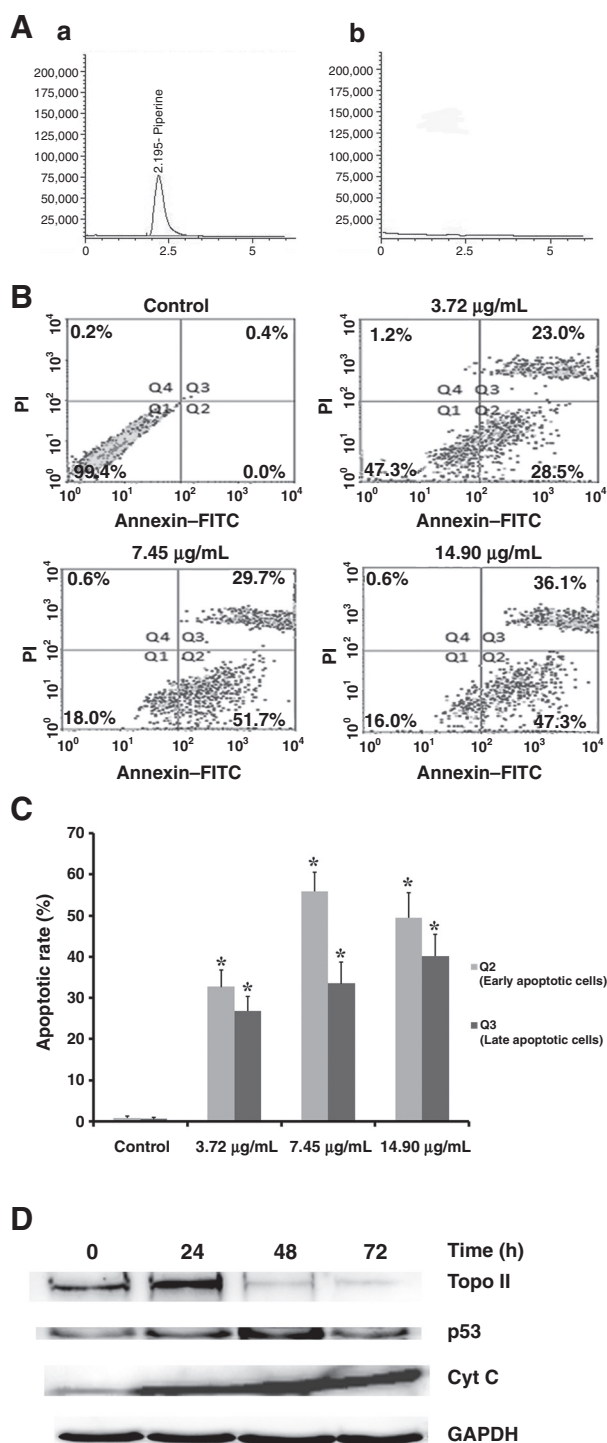


Figure 1. Chromatograms of PFPE and apoptotic induction by PFPE. A, chromatograms of LCMS analysis of piperine: (a) standard of piperine 10 ng/mL, (b) PFPE. B, MCF-7 was treated with 0, 3.72, 7.45, and 14.90 µg/mL of PFPE for 24 hours, and then the induction of apoptosis was measured by flow cytometry. C, histograms showing the distribution of apoptotic cells for three independent experiments. *, $P < 0.05$ indicated statistically significant differences compared with the control. D, effect of PFPE on the topoisomerase II, cyt C, and p53 proteins. MCF-7 cell lines were treated with 7.45 µg/mL of the extract and incubated for 0, 24, 48, and 72 hours.

potent cytotoxic activity against MCF-7 cells and a good selectivity toward the breast cancer cells.

Induction of apoptosis by PFPE in the MCF-7 cell lines

From the cytotoxic results, it was apparent that PFPE was very effective against MCF-7 cells with an IC_{50} value of 7.45 µg/mL. To assess the mechanism of the PFPE-induced cell death, flow cytometry and Western blot analysis were conducted. Staining by FITC-Annexin V and propidium iodide was performed to examine the proapoptotic potential of PFPE. After treatment with 0 (control), 3.72 ($IC_{50}/2$), 7.45 (IC_{50}), and 14.90 ($2IC_{50}$) µg/mL, the percentages of early apoptotic cells (Q2) were $0.8\% \pm 0.5\%$, $32.7\% \pm 4.1\%$, $55.8\% \pm 4.8\%$, and $49.4\% \pm 6.2\%$ of the total cells, respectively. Meanwhile, the late apoptotic cells (Q3) represented $0.6\% \pm 0.4\%$, $26.8\% \pm 3.6\%$, $33.5\% \pm 5.2\%$, and $40.2\% \pm 5.3\%$ of the total cells, respectively (Fig. 1B and C). These results indicated that apoptosis occurred in the MCF-7 cells treated with PFPE in a concentration-dependent manner. The Western blot analysis was used to elucidate the underlying mechanism for the induction of apoptosis by PFPE. The GAPDH protein was used as an internal control. The p53 and cyt C were upregulated, whereas the topo II was downregulated in the MCF-7 cells treated with PFPE at their IC_{50} concentration (Fig. 1D). These data indicated that p53, cyt C, and topo II were all involved in the apoptosis of MCF-7 cell lines induced by PFPE.

Acute toxicity of PFPE in mice

Evaluation of the toxicity profile of PFPE was performed by the acute toxicity study. The study was conducted in mice according to the OECD guideline (18) prior to the *in vivo* anticancer and cancer preventive studies. The mice were given a single dose of the PFPE, orally, at 5,000 mg/kg for the acute toxicity testing. No adverse effects or mortality were observed during 14 days of the acute toxicity testing period. In addition, the treated mice had similar body weights and internal organ weights when compared with the control group. On gross examination, there was no observable tissue damage. Therefore, the results indicated that PFPE could be considered safe for acute exposure at up to 5,000 mg/kg.

Cancer preventive effects of PFPE in NMU-induced mammary tumor rats

This study aimed to determine the cancer preventive ability of PFPE under carcinogenic exposure conditions. The dose of the extract used was determined by a preliminary study on

Table 1. Cytotoxic activity of PFPE on normal breast and breast cancer cell lines

Cell lines	Parameters	
	IC_{50} value \pm SD (μ g/mL) ^a	SI ^b
MCF-7	7.45 \pm 0.60	6.22, 4.54
MDA-MB-468	18.19 \pm 0.59	2.55, 1.86
MDA-MB-231	22.67 \pm 2.31	2.04, 1.49
ZR-75-1	13.85 \pm 1.25	3.34, 2.44
MCF-12A	46.31 \pm 0.76	— ^c
HT-29	27.74 \pm 0.91	1.66, 1.22
SW-620	29.56 \pm 2.47	1.57, 1.14
H-358	34.69 \pm 3.05	1.33, 0.97
A-549	30.77 \pm 1.23	1.51, 1.09
LA-N-5	111.28 \pm 8.85	0.42, 0.30
SK-N-SH	21.51 \pm 1.52	2.15, 1.57
L-929	33.82 \pm 1.12	— ^c

^aThe value obtained from three independent experiments.

^bSI value = (IC_{50} values of MCF-12A/ IC_{50} value of cancer cell lines, IC_{50} values of L-929/ IC_{50} value of cancer cell lines).

^cNot tested.

Table 2. Mean body weight, tumor incidence, tumor weight, and the organ weight/body weight ratio of rats in the study of preventive activity and anticancer activity at sacrifice

Group	Body weight (g)	Tumor incidence (%)	Tumor ^a weight (g)	Organ weight/body weight (%) ^a				
				Heart	Liver	Lung	Spleen	Kidney
Treatment								
Normal	271.33 ± 12.46	—	—	0.37 ± 0.02	3.62 ± 0.19	0.43 ± 0.02	0.27 ± 0.03	0.60 ± 0.06
Control	275.50 ± 12.73	100	5.82 ± 1.38	0.38 ± 0.04	3.84 ± 0.72	0.55 ± 0.11	0.52 ± 0.22	0.71 ± 0.15
Vehicle	269.40 ± 20.07	100	5.02 ± 1.07	0.41 ± 0.06	3.69 ± 0.17	0.52 ± 0.07	0.36 ± 0.16	0.69 ± 0.09
PFPE100	273.50 ± 20.66	100	3.86 ± 0.85 ^{b,c}	0.38 ± 0.05	4.09 ± 0.17	0.50 ± 0.06	0.41 ± 0.16	0.67 ± 0.07
PFPE200	273.56 ± 22.75	100	2.06 ± 0.78 ^{b,c}	0.37 ± 0.04	3.94 ± 0.06	0.53 ± 0.09	0.29 ± 0.06	0.69 ± 0.08
PFPE400	274.50 ± 12.44	100	3.24 ± 0.96 ^{b,c}	0.39 ± 0.07	3.61 ± 0.21	0.53 ± 0.09	0.46 ± 0.22	0.68 ± 0.05
Prevention								
Normal	271.33 ± 12.46	—	NA	0.37 ± 0.02	3.62 ± 0.19	0.43 ± 0.02	0.27 ± 0.03	0.60 ± 0.06
Control	275.50 ± 12.73	100	5.82 ± 1.38	0.38 ± 0.04	3.84 ± 0.72	0.55 ± 0.11	0.52 ± 0.22	0.71 ± 0.15
Vehicle	289.80 ± 24.04	100	3.05 ± 1.52	0.36 ± 0.03	3.35 ± 0.47	0.50 ± 0.07	0.31 ± 0.09	0.65 ± 0.04
PFPE100	290.50 ± 15.30	20	0.95 ± 0.15 ^{b,c}	0.38 ± 0.02	3.08 ± 0.29	0.42 ± 0.03	0.27 ± 0.03	0.64 ± 0.04
PFPE200	294.00 ± 14.80	10	8.2	0.39 ± 0.06	3.48 ± 0.54	0.49 ± 0.05	0.32 ± 0.10	0.64 ± 0.06

^a*P* < 0.05, significantly different compared with the normal group.

^b*P* < 0.05, significantly different compared with the control group.

^c*P* < 0.05, significantly different compared with the vehicle group.

NMU-induced mammary tumorigenesis in rats (data not shown). Rats were treated with PFPE at doses of 100 and 200 mg/kg after the first application of NMU. After 76 days of treatment, the incidences of rats bearing tumors were 20% and 10% in the 100 mg/kg and 200 mg/kg of PFPE-treated groups, respectively. In contrast, a 100% incidence was found in the control and vehicle groups. There were no significant differences in organ size between the PFPE-treated groups and the vehicle group (Table 2). However, the tumor size of the PFPE-treated group at 100 mg/kg had a much slower growth when compared with the control and vehicle groups, whereas the PFPE-treated group at 200 mg/kg grew well. The hematologic and clinical chemistry values of the PFPE-treated rats were not significantly changed when compared with the control and vehicle groups as shown in Table 3.

Effect of PFPE against NMU-induced mammary tumorigenesis in rats

The rationale of this experiment was to examine the anticancer properties of PFPE on chemically induced breast cancer. For induction of malignant mammary tumors, all rats were injected with three intraperitoneal doses of NMU, one at 50 and then at 80 and 110 days of age. The mean day that tumors were first observed was 119.09 ± 9.42 days. The tumor size of the treated group with PFPE at the doses of 100, 200 and 400 mg/kg was significantly reduced when compared with the control and vehicle groups. In addition, the internal organ weight/body weight ratios were not significantly changed when compared with the normal, control, and vehicle groups (Table 2). The histologic results of all organs in the PFPE-treated groups were normal.

Table 3. Hematologic values and clinical chemistry values of the preventive study on the induced mammary tumorigenesis in rats

	Normal	Control	Vehicle	PFPE	
				100 mg/kg	200 mg/kg
Hematologic values					
White blood cells ($\times 10^3/\mu\text{L}$)	2.90 ± 0.81	3.15 ± 1.71	3.45 ± 0.30	2.58 ± 0.38	3.60 ± 0.70
Neutrophil (%)	85.25 ± 4.83	73.13 ± 12.17	79.17 ± 2.06	83.00 ± 1.69	74.63 ± 3.29
Lymphocyte (%)	14.75 ± 4.83	25.75 ± 11.94	17.67 ± 2.20	15.60 ± 1.32	22.75 ± 2.68
Monocyte (%)	0.00 ± 0.00	0.50 ± 1.07	2.33 ± 0.42	1.20 ± 0.80	1.88 ± 1.01
Eosinophil (%)	0.00 ± 0.00	0.62 ± 0.92	0.50 ± 0.22	0.20 ± 0.13	0.75 ± 0.41
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Red blood cells ($\times 10^6/\mu\text{L}$)	6.82 ± 0.36	6.29 ± 0.50	6.36 ± 0.27	6.43 ± 0.14	6.28 ± 0.20
Hemoglobin (g/dl)	13.79 ± 0.51	13.60 ± 0.90	13.52 ± 0.52	13.46 ± 0.33	13.10 ± 0.42
Hematocrit (%)	38.53 ± 1.74	36.21 ± 3.05	37.22 ± 1.61	36.60 ± 0.90	36.53 ± 0.74
Mean corpuscular volume (fl)	56.55 ± 1.04	59.35 ± 2.62	58.55 ± 0.37	56.86 ± 0.34	58.35 ± 0.90
Mean corpuscular hemoglobin (pg)	20.25 ± 0.61	21.45 ± 0.69	21.32 ± 0.25	20.91 ± 0.20	20.86 ± 0.18
Mean corpuscular hemoglobin concentration (g/dl)	35.82 ± 0.78	36.78 ± 1.09	35.71 ± 0.34	36.78 ± 0.20	35.81 ± 0.55
Platelet ($\times 10^5/\mu\text{L}$)	546.75 ± 59.88	512.63 ± 96.94	522.33 ± 30.76	527.00 ± 18.01	510.02 ± 18.79
Clinical blood chemistry values					
BUN (mg/dl)	22.93 ± 3.76	32.04 ± 10.04	26.43 ± 1.21	22.92 ± 0.50	27.43 ± 1.66
Creatinine (mg/dl)	0.57 ± 0.13	0.52 ± 0.13	0.62 ± 0.07	0.54 ± 0.03	0.61 ± 0.04
Total protein (g/dl)	6.77 ± 0.47	5.57 ± 0.36	6.34 ± 0.21	6.61 ± 0.14	6.51 ± 0.11
Albumin (g/dl)	3.42 ± 0.20	3.11 ± 0.24	3.35 ± 0.05	3.35 ± 0.04	3.34 ± 0.05
Total bilirubin (mg/dl)	0.20 ± 0.11	0.33 ± 0.14	0.32 ± 0.09	0.21 ± 0.04	0.30 ± 0.06
SGOT (U/L)	109.83 ± 21.14	179.38 ± 31.56	105.17 ± 8.16	112.50 ± 6.58	116.88 ± 9.91
SGPT (U/L)	55.00 ± 17.72	56.38 ± 11.35	60.33 ± 9.04	48.70 ± 2.60	48.38 ± 6.51
Alkaline phosphatase (U/L)	82.33 ± 20.42	123.88 ± 30.12	102.17 ± 9.37	108.90 ± 15.75	116.38 ± 19.59

Abbreviations: SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

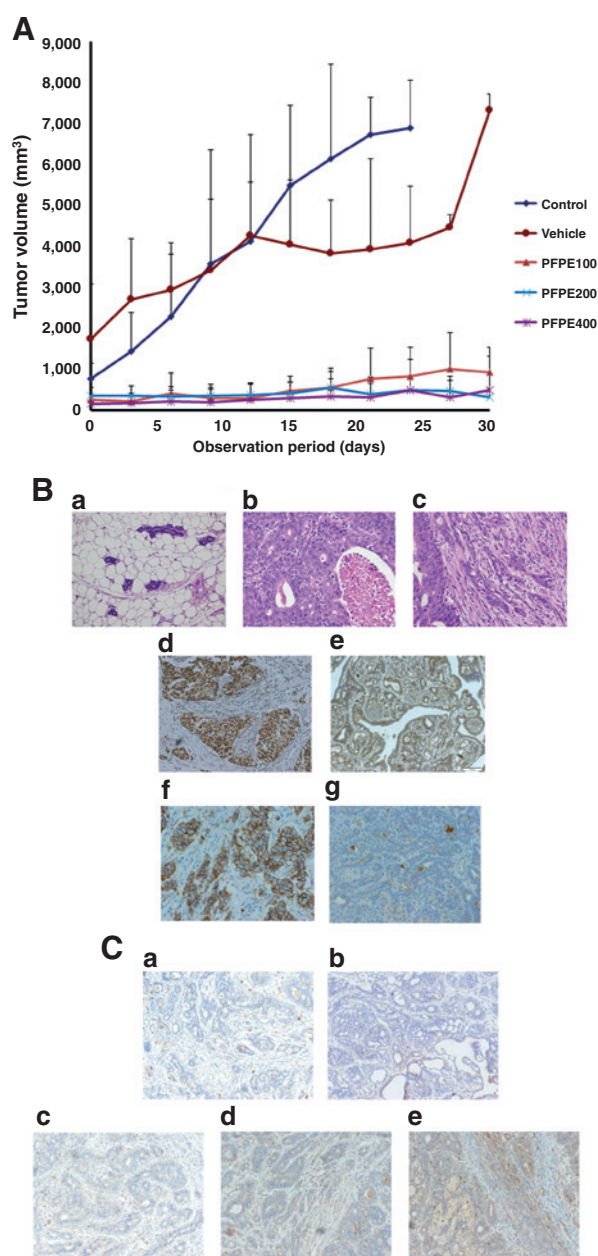


Figure 2. Effects of PFPE treatments in NMU-induced tumorigenesis in rats. A, the mean tumor volume of NMU-induced tumorigenesis rats during the observation period. B, classification of tumors in the NMU-induced mammary tumorigenesis rats: (a) adjacent normal tissue, (b) ductal carcinoma *in situ*, (c) invasive ductal carcinoma. The pattern of ER staining on histology sections: (d) positive control, (e) rat tumor; and the pattern of HER-2 staining on histology sections: (f) positive control, (g) rat tumor (200 \times). C, immunohistochemical staining of cyt C: (a) control, (b) vehicle, (c) PFPE 100 mg/kg, (d) PFPE 200 mg/kg and (e) PFPE 400 mg/kg (100 \times).

The mean tumor volume during the observation period is shown in Fig. 2A. The tumor volume was dramatically increased in the control and vehicle groups. It was a surprise that the tumor volumes in all the PFPE-treated groups were slightly increased

when compared with the initial volumes after 30 days of treatment. The results indicated a good response of the tumors to the extract. All tumors observed after sacrifice were encapsulated and identified by tumor histology into two types, ductal carcinoma *in situ* and invasive ductal carcinoma (Fig. 2Ba–Bc). The patterns of the ER and HER-2 staining on histologic sections are shown in Fig. 2B. The tumors were characterized as being ER-positive (Fig. 2Be) and HER-2-negative (Fig. 2Bg) by comparison with the positive control samples (Fig. 2Bd and Bf). In addition, the immunohistochemical staining of the rat tumor directed against cyt C is shown in Fig. 2Ca–Ce. The samples in the PFPE-treated groups exhibited cyt C staining that was higher than 10% (Fig 2Cc–Ce). However, the cyt C staining of tumors was lower than 10% in the control and vehicle groups (Fig 2Ca–Cb). The intensity of cyt C staining was strongest in the group treated with 400 mg/kg of PFPE.

There was no significant change in the hematologic and clinical chemistry values in the group treated with PFPE when compared with the normal, control, and vehicle groups (Table 4). However, the values of the doxorubicin-treated group were significantly changed in white blood cell, Red blood cell count, hemoglobin, hematocrit, blood urea nitrogen (BUN), albumin, total protein, total bilirubin, and alkaline phosphatase when compared with the normal, control, and vehicle groups.

Discussion

Many natural resources have been used to detect anticancer drugs. Previous studies have revealed that compounds isolated from plants of the genus *Piper* had potent anticancer properties (13, 21–24). The results of the present study illustrated that the PFPE had anticancer and cancer preventive effects on mammary tumorigenesis in rats.

Many natural pure compounds have been found in the genus *Piper*, the major ones being alkaloids, terpenes, and polyphenols (22). The pepper alkaloids are also present in the dried fruits of *P. nigrum*, piperine, and piperlongumine and have exhibited cytotoxic effects on HL-60, CEM, HCT-8, and B-16 cell lines (23). In addition, *P. nigrum* and its derivative extracts have been reported to inhibit tumor growth *in vitro* and *in vivo* (13, 16, 24). While screening for cytotoxic effects of plants of the genus *Piper* in our previous study, we found that the recrystallized extract of *P. nigrum* that was piperine free had a potent cytotoxic activity on breast cancer cell lines. The absence of piperine was confirmed by using LC/MS. In addition, the extract contained alkaloids using the Dragendorff's reagent for testing (16). Alkaloids often possess potent cytotoxicity to many cells, and some have been used as anticancer agents. In this study, the antiproliferative effect of PFPE was examined on four breast cancer cell lines. This crude alkaloid extract had a cytotoxic effect against luminal MCF-7 and ZR-75-1 cells with an SI value that was higher than for the basal-like MDA-MB-231 and MDA-MB-468 cells. The extract inhibited proliferation of luminal breast cancer cells more than the basal-like breast cancer cells. Based on ER/HER-2/PR gene expressions, five distinct subtypes of breast cancer have been defined. The six intrinsic subtypes included the ER-positive luminal A and luminal B, the ER-positive basal-like, HER2-enriched and normal breast, and claudin-low. The luminal tumors were also more sensitive to chemotherapy than the basal types (25). A recent retrospective study of 1,134 breast cancer subjects showed that luminal-like (ER-positive tumor) breast tumors occurred at a higher

Table 4. Hematologic values and clinical chemistry values of the study of anticancer activity on induced mammary tumorigenesis in rats

	Normal	Control	Vehicle	PFPE		
				100 mg/kg	200 mg/kg	400 mg/kg
Hematologic values						
White blood cells ($\times 10^3/\mu\text{L}$)	2.90 \pm 0.81	3.15 \pm 1.71	4.28 \pm 1.97	5.35 \pm 2.94	3.17 \pm 0.95	6.51 \pm 3.79
Neutrophil (%)	85.25 \pm 4.83	73.13 \pm 12.17	67.90 \pm 14.32	77.70 \pm 11.50	74.78 \pm 10.66	75.71 \pm 7.09
Lymphocyte (%)	14.75 \pm 4.83	25.75 \pm 11.94	29.30 \pm 12.89	21.50 \pm 11.27	23.33 \pm 8.80	23.29 \pm 6.53
Monocyte (%)	0.00 \pm 0.00	0.50 \pm 1.07	2.50 \pm 2.37	0.30 \pm 0.48	0.78 \pm 0.97	1.00 \pm 1.82
Eosinophil (%)	0.00 \pm 0.00	0.62 \pm 0.92	0.30 \pm 0.48	0.50 \pm 0.85	1.11 \pm 1.36	0.00 \pm 0.00
Basophil (%)	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
Red blood cells ($\times 10^6/\mu\text{L}$)	6.82 \pm 0.36	6.29 \pm 0.50	5.99 \pm 1.23	6.47 \pm 1.30	6.67 \pm 1.26	5.38 \pm 1.95
Hemoglobin g/dl)	13.79 \pm 0.51	13.60 \pm 0.90	12.63 \pm 2.16	13.49 \pm 2.65	13.81 \pm 2.52	11.77 \pm 3.72
Hematocrit (%)	38.53 \pm 1.74	36.21 \pm 3.05	35.31 \pm 5.70	38.30 \pm 7.71	38.94 \pm 6.89	32.83 \pm 9.88
Mean corpuscular volume (fl)	56.55 \pm 1.04	59.35 \pm 2.62	59.71 \pm 5.97	58.15 \pm 1.08	58.52 \pm 1.98	64.46 \pm 11.44
Mean corpuscular hemoglobin (pg)	20.25 \pm 0.61	21.45 \pm 0.69	21.29 \pm 1.74	20.62 \pm 0.67	20.76 \pm 0.88	22.83 \pm 3.19
Mean corpuscular hemoglobin concentration (g/dl)	35.82 \pm 0.78	36.78 \pm 1.09	35.71 \pm 1.07	35.47 \pm 0.87	35.42 \pm 1.02	35.59 \pm 1.84
Platelet ($\times 10^5/\mu\text{L}$)	546.75 \pm 59.88	512.63 \pm 96.94	522.90 \pm 107.51	440.20 \pm 79.52	508.89 \pm 172.14	520.02 \pm 112.47
Clinical blood chemistry values						
BUN (mg/dl)	22.93 \pm 3.76	32.04 \pm 10.04	25.49 \pm 2.88	25.31 \pm 6.81	21.47 \pm 2.82	28.21 \pm 4.95
Creatinine (mg/dl)	0.57 \pm 0.13	0.52 \pm 0.13	0.52 \pm 0.10	0.48 \pm 0.05	0.47 \pm 0.07	0.50 \pm 0.14
Total protein (g/dl)	6.77 \pm 0.47	5.57 \pm 0.36	5.78 \pm 0.62	6.04 \pm 0.50	6.29 \pm 0.26	5.82 \pm 0.79
Albumin (g/dl)	3.42 \pm 0.20	3.11 \pm 0.24	3.24 \pm 0.18	3.21 \pm 0.28	3.48 \pm 0.16	3.11 \pm 0.31
Total bilirubin (mg/dl)	0.20 \pm 0.11	0.33 \pm 0.14	0.27 \pm 0.05	0.28 \pm 0.06	0.39 \pm 0.14	0.32 \pm 0.14
SGOT (U/L)	109.83 \pm 21.14	179.38 \pm 31.56	136.89 \pm 26.11	125.20 \pm 34.74	127.89 \pm 71.51	137.86 \pm 34.29
SGPT (U/L)	55.00 \pm 17.72	56.38 \pm 11.35	56.89 \pm 11.92	46.57 \pm 9.95	55.00 \pm 14.34	54.71 \pm 12.72
Alkaline phosphatase (U/L)	82.33 \pm 20.42	123.88 \pm 30.12	136.22 \pm 27.23	105.00 \pm 47.58	88.00 \pm 12.02	118.86 \pm 38.05

Abbreviations: SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

prevalence. Also of note, the 5-year overall survival rate in patients with luminal breast cancer was greater than 80% (26). Therefore, the results on the breast cancer cell lines supported the use of this extract as an anticancer agent in luminal-like breast cancer patients. In addition, the results showed that PFPE exhibited selectivity.

In order to elucidate the mechanism of the antiproliferative activity, the apoptosis effect of PFPE on MCF-7 cells was investigated using flow cytometry and Western blot analysis. A significant increase of the early and late apoptotic cells was observed in the MCF-7 cells treated with PFPE for 24 hours in a concentration-dependent manner. Furthermore, many alkaloids with an anticancer effect, such as ellipticine and etoposide, are topoisomerase inhibitors that cause damage to DNA. Moreover, a catalytic inhibitor of DNA topoisomerase II, merbarone, has previously been reported to generate DNA damage (27). It can induce p53 levels to serve as a cell-cycle checkpoint. The expression of p53 was increased by the topoisomerase-inhibiting etoposide and led to apoptosis in human embryonic stem cells (28). In addition, p53 is a DNA-binding transcription factor that activates genes responsible for apoptosis, such as Bax and PUMA (29, 30), and downregulates the expression of the topoisomerase II α promoter (31). Bax and PUMA can trigger the release of cyt C, an intermediate in the apoptosis pathway, from mitochondria (32–34). The present results demonstrated that the topoisomerase II was downregulated, causing DNA damage in the MCF-7 treated with PFPE for 72 hours in a time-dependent manner. The expression of the p53 and cyt C levels was decreased when compared with the untreated cells in response to DNA damage. Accordingly, the apoptotic effects of PFPE may result in DNA damage. Based on the results, we propose that the extract could be an anticancer agent.

To test for the anticancer and cancer preventive effects of PFPE, an *in vivo* mammary tumor model induced in rats by NMU was used. These carcinogen-induced rat tumors are thought to have mutations in the *H-ras* oncogene at codon 12 (35). Treck and

colleagues have provided evidence that the *H-ras* protein has an important role in estrogenic epidermal growth factor effects in breast cancer cell lines (36). With regard to the tumor characterization, the histologic results showed that there were two types of breast carcinoma, ductal carcinoma *in situ* and invasive ductal carcinoma. Furthermore, the tumors have been shown to be ER-positive by the immunohistochemical assay, which is in agreement with Chan's study (37), but are HER-2 negative. Hence, the tumors in the NMU-induced mammary tumorigenesis rats appeared to be closely similar to those of the human luminal-like breast cancer. Results from the cancer preventive study showed a marked decrease in the incidence of the NMU-induced tumorigenesis in rats treated with PFPE. The tumor size was also reduced in the group treated with 100 mg/kg of the extract but was not reduced in a rat treated with 200 mg/kg of PFPE. No significant damage and weight loss of internal organs were detected in any of the treatment groups. In addition, the tumor incidence and multiplicity of rats in PFPE-treated groups were lower than for the vehicle group. These results indicated that PFPE had a cancer preventive effect on NMU-induced mammary tumorigenesis in rats.

For the anticancer study, the tumor sizes in rats treated with the PFPE were significantly reduced when compared with the control and vehicle groups. The treatment of PFPE was found not to produce any change in food consumption or other adverse reactions in rats. Our results demonstrated that PFPE had a strong anticancer activity on mammary tumorigenesis in rats, with no apparent adverse effects. For the immunohistochemical staining of tumors, we found that the increase of expression of cyt C in the PFPE-treated groups correlated well with the finding in Western blot analysis. The intensity of the cyt C staining of tumors was increased in a dose-dependent manner. These results indicated that PFPE induced apoptosis in breast cancer cells. The preventive activity and anticancer activity studies produced results that corroborated the findings with MTT. Therefore, *P. nigrum*

extracts deserve further investigation to understand the mechanism of action of its constituents for therapeutic applications to treat cancer.

Conclusion

The PFPE exhibited potent cytotoxic effects against MCF-7 breast cancer cells that were mediated through the induction of the apoptotic pathway. In addition, the extract had cancer preventive and anticancer effects on mammary tumorigenesis in rats. Moreover, PFPE did not affect liver and bone marrow. Nevertheless, the pure compounds isolated from PFPE, which induced apoptosis on breast cancer cells, need to be identified in further studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Sriwiryajan, P. Graidist
Development of methodology: S. Sriwiryajan, N. Lailerd, P. Graidist
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Sriwiryajan, P. Graidist
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Sriwiryajan, P. Graidist

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