View article

scholar.google.co.th/citations?view_op=view_citation&hl=en&user=ZahA7tYAAAAJ&citation_for_view=ZahA7tYAAAAJ:IjCSPb-OGe4C

View article

Araya Khoka

Edit Delete

Anticancer effects of piperine-free Piper nigrum extract on cholangiocarcinoma cell lines

[HTML] from phcog.com
Full View

Authors	Aman Tedasen, Araya Khoka, Siribhorn Madla, Somchai Sriwiriyajan, Potchanapond Graidist
Publication date	2020/3/1
Journal	Pharmacognosy Magazine
Volume	16
Issue	68
Pages	28
Publisher	Medknow Publications
Description	Background: Black pepper (Piper nigrum L.) is widely used as a traditional medicine, including usage for pain relief, fevers, as well as an anticancer agent. Previously, we reported that piperine-free P. nigrum extract (PFPE) inhibited breast cancer in vitro and in vivo. Objective: In this present study, we explored the anticancer effects of PFPE on cholangiocarcinoma (CCA). Materials and Methods: 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was performed to analyze cytotoxic potential of PFPE whereas deoxyribonucleic acid (DNA) fragmentation

Publisher Medknow Publications

Description

Background:

Black pepper (Piper nigrum L.) is widely used as a traditional medicine, including usage for pain relief, fevers, as well as an anticancer agent. Previously, we reported that piperine-free P. nigrum extract (PFPE) inhibited breast cancer in vitro and in vivo.

Objective:

In this present study, we explored the anticancer effects of PFPE on cholangiocarcinoma (CCA).

Materials and Methods:

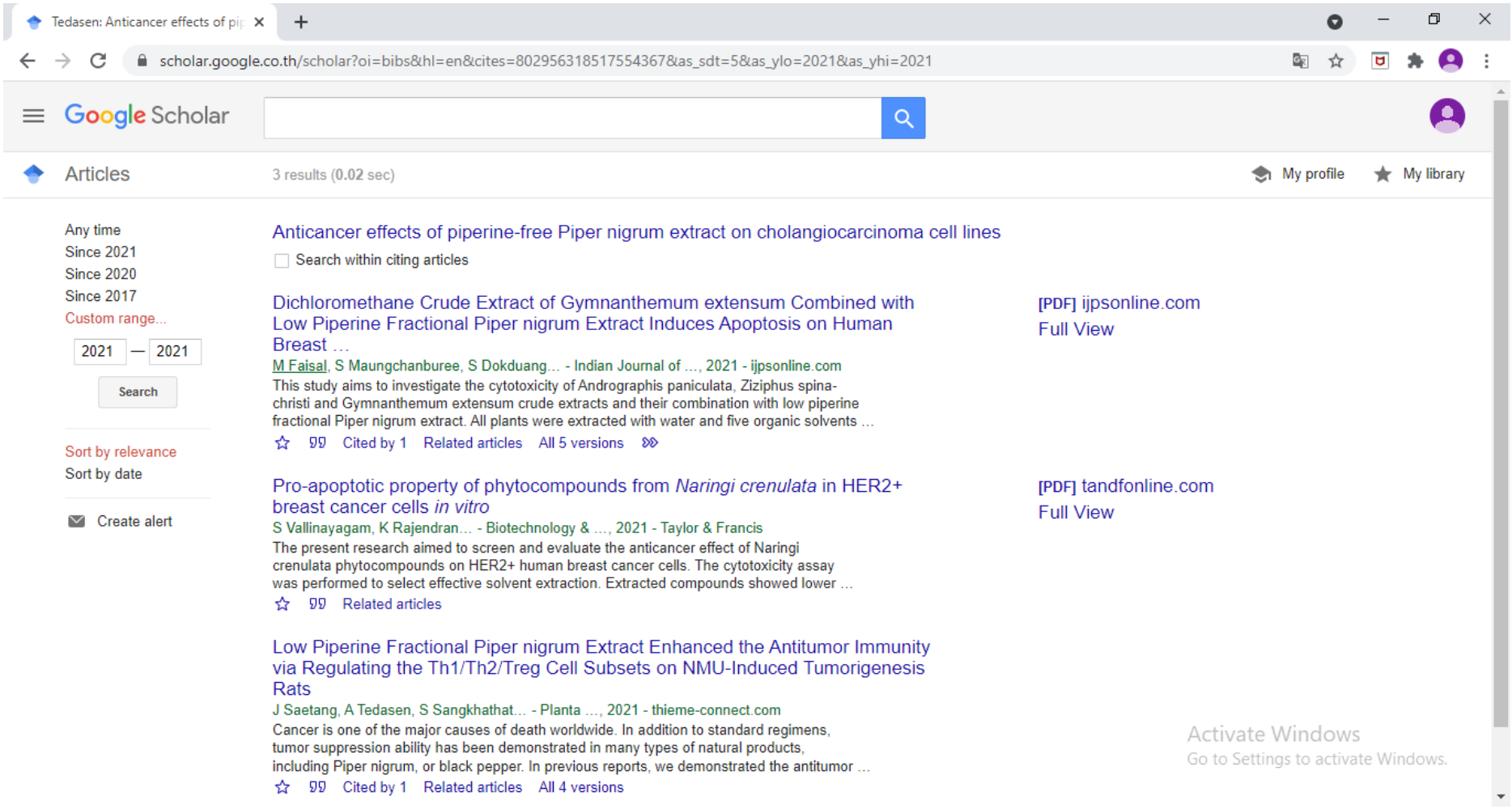
3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was performed to analyze cytotoxic potential of PFPE whereas deoxyribonucleic acid (DNA) fragmentation followed by Western blot analysis were used.

Results:

PFPE composed of alkaloid, flavonoid, amide, lignans, opioid, and steroid. This crude extract represented cytotoxic effect against CCA cells which stronger than dichloromethane P. nigrum crude extract and piperine, especially on KKU-M213 (median inhibition concentration [IC 50] at 13.70 μg/ml ...

Total citations Cited by 3

Scholar articles Anticancer effects of piperine-free Piper nigrum extract on cholangiocarcinoma cell lines
A Tedasen, A Khoka, S Madla, S Sriwiriyajan... - Pharmacognosy Magazine, 2020
Cited by 3 Related articles All 4 versions


Tedasen: Anticancer effects of pi
scholar.google.co.th/scholar?oi=bibs&hl=en&cites=8029563185175543678as_sdt=5&as_ylo=2021&as_yhi=2021
Google Scholar
Articles
3 results (0.02 sec)
My profile
My library
Any time
Since 2021
Since 2020
Since 2017
Custom range...
2021 — 2021
Search
Sort by relevance
Sort by date
Create alert
Anticancer effects of piperine-free Piper nigrum extract on cholangiocarcinoma cell lines
Search within citing articles
Dichloromethane Crude Extract of Gymnanthemum extensum Combined with Low Piperine Fractional Piper nigrum Extract Induces Apoptosis on Human Breast …
M Faisal, S Maungchanburee, S Dokduang… - Indian Journal of …, 2021 - ijpsonline.com
This study aims to investigate the cytotoxicity of Andrographis paniculata, Ziziphus spina-christi and Gymnanthemum extensum crude extracts and their combination with low piperine fractional Piper nigrum extract. All plants were extracted with water and five organic solvents …
Cited by 1
Related articles
All 5 versions
[PDF] ijpsonline.com
Full View
Pro-apoptotic property of phytocompounds from Naringi crenulata in HER2+ breast cancer cells in vitro
S Vallinayagam, K Rajendran… - Biotechnology & …, 2021 - Taylor & Francis
The present research aimed to screen and evaluate the anticancer effect of Naringi crenulata phytocompounds on HER2+ human breast cancer cells. The cytotoxicity assay was performed to select effective solvent extraction. Extracted compounds showed lower …
Related articles
[PDF] tandfonline.com
Full View
Low Piperine Fractional Piper nigrum Extract Enhanced the Antitumor Immunity via Regulating the Th1/Th2/Treg Cell Subsets on NMU-Induced Tumorigenesis Rats
J Saetang, A Tedasen, S Sangkhathat… - Planta …, 2021 - thieme-connect.com
Cancer is one of the major causes of death worldwide. In addition to standard regimens, tumor suppression ability has been demonstrated in many types of natural products, including Piper nigrum, or black pepper. In previous reports, we demonstrated the antitumor …
Cited by 1
Related articles
All 4 versions
Activate Windows
Go to Settings to activate Windows.

Low Piperine Fractional *Piper nigrum* Extract Enhanced the Antitumor Immunity via Regulating the Th1/Th2/Treg Cell Subsets on NMU-Induced Tumorigenesis Rats

Authors
Jirakrit Saetang[1,2,4], Aman Tedasen[1,3], Surasak Sangkhathat[2], Natnaree Sangkaew[1], Sirinapa Dokduang[1,3], Napat Prompat[1], Siriporn Taraporn[1,3], Potchanapond Graidist[1,3]

Affiliations
1 Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand
2 Department of Surgery, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand
3 The Excellent Research Laboratory of Cancer Molecular Biology, Prince of Songkla University, Songkhla, Thailand
4 EZ-Mol-Design Laboratory, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand

Key words
Piperaceae, *Piper nigrum*, antitumor immunity, Th1, Th2, Treg, PFPE

received December 16, 2020
accepted after revision March 20, 2021
published online

Bibliography
Planta Med 2021
DOI 10.1055/a-1458-5646
ISSN 0032-0943

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Correspondence
Assoc. Prof. Dr. Potchanapond Graidist, PhD
Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University
15 Karnjanavanich Rd., Songkhla, 90110, Thailand
Phone: + 66 74 45 11 80, Fax: + 66 74 42 95 84
gpotchan@medicine.psu.ac.th

Supplementary material is available under https://doi.org/10.1055/a-1458-5646

ABSTRACT

Cancer is one of the major causes of death worldwide. In addition to standard regimens, tumor suppression ability has been demonstrated in many types of natural products, including *Piper nigrum*, or black pepper. In previous reports, we demonstrated the antitumor effect of low piperine fractional *Piper nigrum* extract *in vitro* and *in vivo*. However, the effects of low piperine fractional *P. nigrum* extract in the aspect of antitumor immunity has not yet been investigated. In this study, tumor-bearing rats were fed with 100 mg/kg BW or 200 mg/kg BW of low piperine fractional *P. nigrum* extract 3 times per week for 4 weeks. Tumor burden and hematological data were then evaluated. Immunological data was investigated using a cytokine array and flow cytometry. The results showed that both doses of low piperine fractional *P. nigrum* extract significantly suppressed tumor progression in N-nitrosomethylurea-induced mammary tumor rats. There were no significant changes observed in the total white blood cells, red blood cells, and hemoglobin. Low piperine fractional *P. nigrum* extract suppressed some cytokine and chemokine levels including CXCL7, sICAM-1, and L-selectin 0.2- to 0.6-fold. Interestingly, 200 mg/kg BW of low piperine fractional *P. nigrum* extract significantly promoted type 1 T helper cell, and suppressed neutrophil, basophil, type 2 T helper cell, and regulatory T cell compared to the control group. In summary, these results indicate that low piperine fractional *P. nigrum* extract had a high efficacy in supporting antitumor activity at immunological levels via regulating Th1/Th2/Treg cells.

Introduction

Black pepper, or *Piper nigrum*, is a medicinal plant in the Piperaceae family. This plant has been recognized for its antioxidant [1], antimicrobial [2], and gastroprotective activities [3]. Major compounds found in *P. nigrum* extract are alkaloids, including piperine, chavicine, piperidine, and piperetine, while terpenes, steroids, lignans, flavones, and alkamides are other dominant substances [4]. Interestingly, the extract from *P. nigrum* showed an inhibitory effect in many cancer models, including breast [5], colon [6], and cervical [7]. Among other constituents, piperine has been widely studied and demonstrated its role as an anticancer property through antiproliferative and anti-inflammation effects [8]. However, toxicity in the reproductive system of piperine was reported in animal models [9]. Another unsatisfac-

▶ **Table 1** Phytochemical ranking associated with antitumor, antioxidant, and anti-inflammation in PFPE.

Ranking	Names	Chemical formulas	Peak area %	Activity
1	Caryophyllene	$C_{15}H_{24}$	25.02	Antioxidant, anticancer, antibacterial, and antifungal activities [21]
2	3-Carene	$C_{10}H_{16}$	3.50	Antioxidant and anticancer activities [22]
3	Pellitorine	$C_{14}H_{25}NO$	2.50	Anticancer and anti-inflammation [23]
4	beta-Selinene	$C_{15}H_{24}$	2.42	Antioxidant activity [24]
5	Pipersintenamide	$C_{19}H_{23}NO_3$	2.12	Cytotoxic effect against CCRF-CEM, HL-60, PC-3, and HA22T cell lines [25]
6	alpha-Humulene	$C_{15}H_{24}$	2.04	Anti-inflammation [26]
7	Guineesine	$C_{24}H_{33}NO_3$	1.63	Anti-inflammation [27]
8	Piperlonguminine	$C_{16}H_{19}NO_3$	1.63	Anticancer [28]
9	beta-Elemene	$C_{15}H_{24}$	1.60	Anticancer [29]
10	2(10)-Pinene	$C_{10}H_{16}$	1.54	Anticancer and anti-inflammation [30]
11	beta-Bisabolene	$C_{15}H_{24}$	1.48	Anticancer [31]
12	Caryophyllene oxide	$C_{15}H_{24}O$	1.16	Antioxidant, anticancer, antibacterial, and antifungal activities [21]
13	Kusunokinin	$C_{21}H_{22}O_6$	1.05	Anticancer [32]
14	Piperyline	$C_{16}H_{17}NO_3$	0.92	Anticancer [33]
15	delta-Elemene	$C_{15}H_{24}$	0.78	Anticancer [34]

tory effect is that piperine may increase the stability of the Aspergillus carcinogenic toxin aflatoxin B1 in the liver [10].

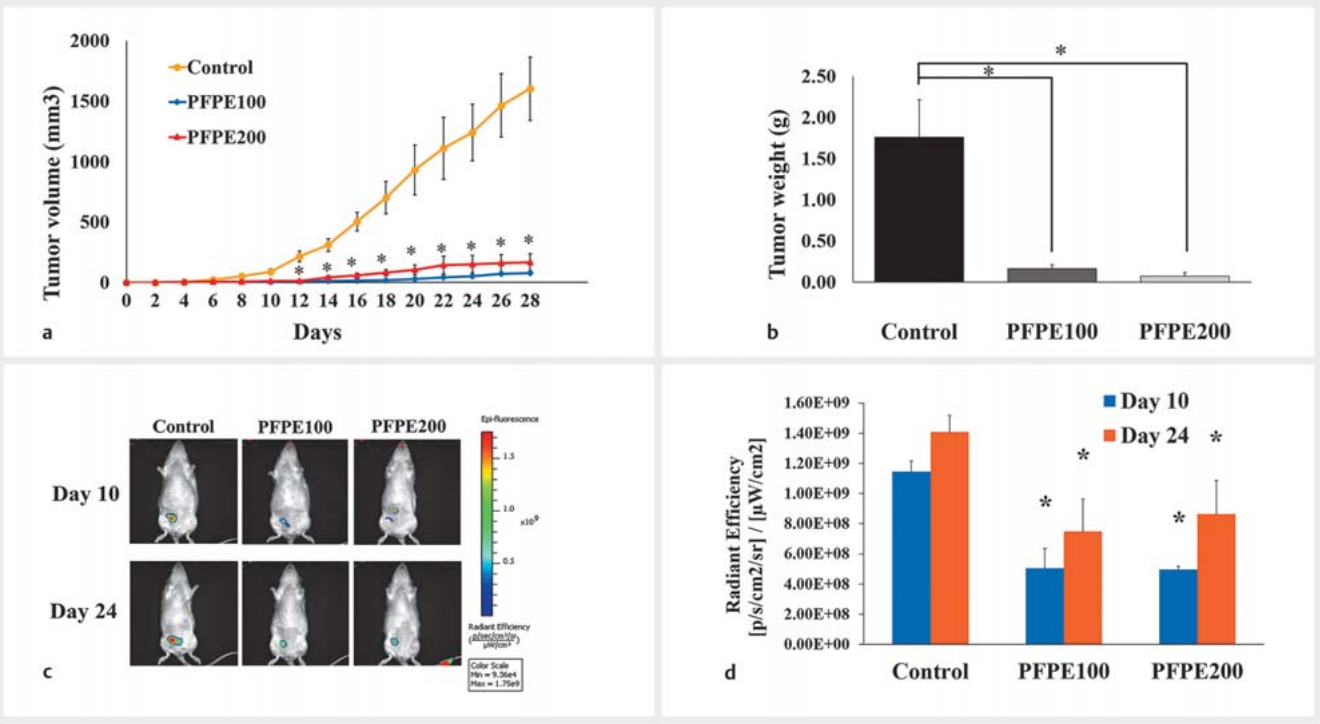
Human immune surveillance is a useful process of the body that suppress the development of tumorigenesis. However, the immune response is also a factor that drives cancer cells into the escape phase, leading to the clinical appearance of cancer [11]. To overcome cancer escape, the strategies termed cancer immunotherapy have been developed trying to use certain immune cells or components to boost anticancer immune response, especially type 1 T helper (Th1) cells or cytotoxic T lymphocytes. Interestingly, the immune boosting property against cancer of some natural compounds and derivatives from plants have been reported. For example, curcumin, the compound extracted from *Curcuma longa*, promoted the production of interferon-γ (IFN-γ) and the polarization of Th1 cells in lung cancer patients [12]. Other natural products, including *Scutellaria ocmulgee* leaf extract [13] and green tea extract [14], also demonstrated an immunomodulation effect on anticancer immunity.

Many reports have shown the immunoregulatory ability of black pepper both *in vitro* and *in vivo*, especially the effect on T cell subtype activation and cytokine expression. In the allergic rhinitis model, mice receiving *P. nigrum* extract orally displayed an improvement of allergic nasal symptoms with the increasing level of Th1 cytokines and a decrease of Th2 and Th17 cytokines [15]. Another allergic model found consistent results with the fact that black pepper extract regulated the homeostasis of cytokine production in bronchoalveolar lavage fluid and lung tissue [16]. Moreover, *P. nigrum* extract affected cytokine production in splenocytes via the release of IFN-γ, a Th1 cytokine, and the suppression of IL-4, a Th2 cytokine [17]. This plant extract also enhanced the cytotoxicity of natural killer cells, which indicates its antitumor immune boosting ability [17].

Previously, low piperine fractional *P. nigrum* extract (PFPE) was shown to have a stronger antitumor effect compared with the piperine compound on breast cancer and cholangiocarcinoma cell lines [18, 19]. PFPE induced breast cancer cell death through the induction of apoptosis in N-nitrosomethylurea (NMU)-induced mammary tumorigenesis rats [20]. Although the anticancer mechanism of PFPE was reported through an apoptosis-inducing effect with low toxicity, the effect of PFPE on the antitumor immune response is still unclear. In this work, we evaluated the number of Th1, Th2, and regulatory T cells (Treg) in mammary tumorigenesis rats treated with PFPE. Moreover, the cytokine array was used to determine the interaction of PFPE on cytokines' response.

Results

The obtained PFPE was sticky and oily with a black color in appearance, with a yield of 2.2% from the initial black peppercorns. Accordingly, the GC-MS analysis revealed that more than 100 compounds were alkaloids, terpenes, amides, and lignans (**Table S1**, Supporting Information). The top 15 phytochemicals contributing to antioxidant, antibacterial, antifungal, and anticancer activities were approximately 49% (▶ **Table 1**). Caryophyllene (25.02%) was the major constituent in PFPE. Carene existed as the second major compound with 3.5%. Moreover, pellitorine, β-selinene, α-humulene, and pipersintenamide were the third major components, each exhibiting around 2–2.5% of the peak area. The other

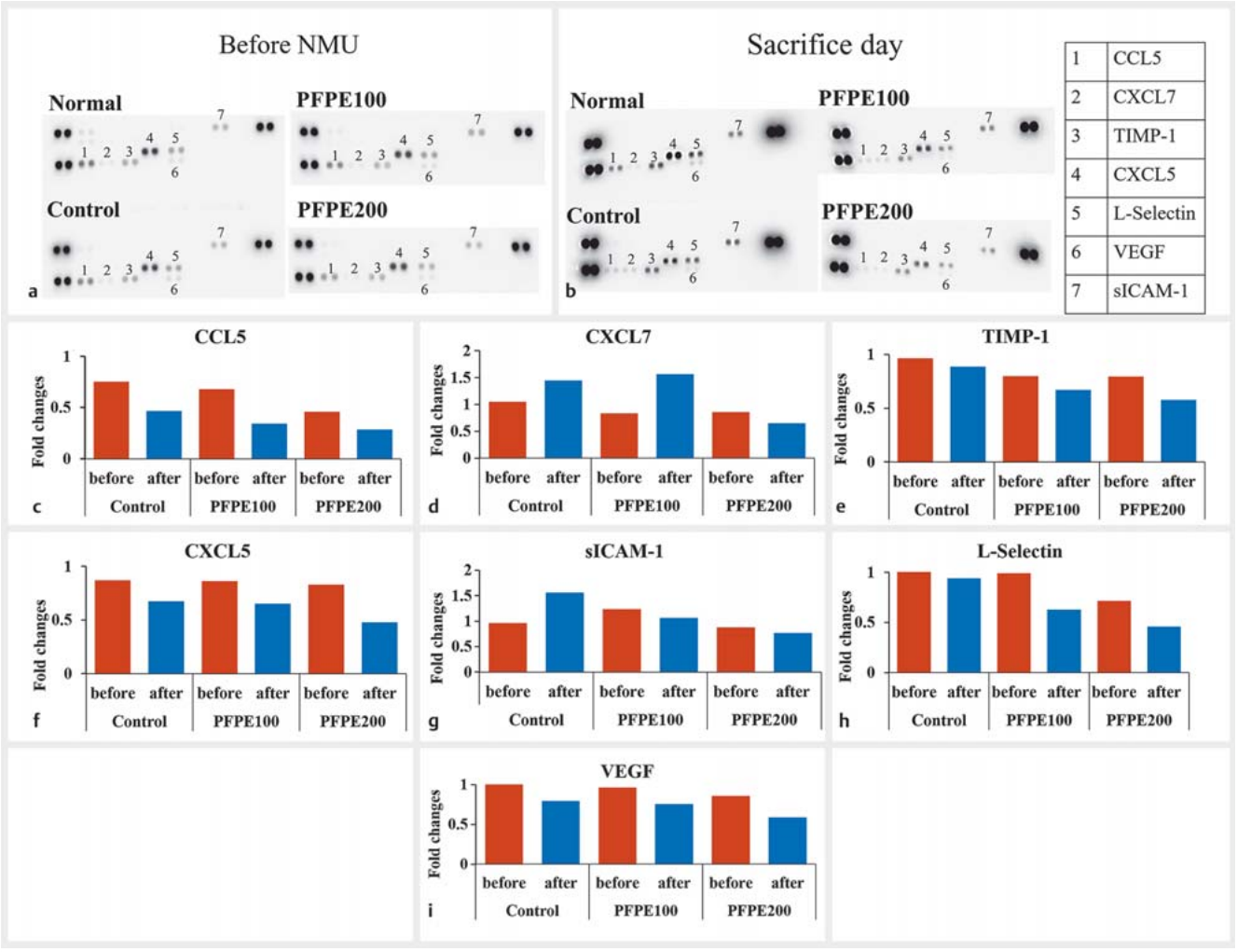


▶ **Fig. 1** Anticancer activity of PFPE in NMU-induced mammary tumor rats. After tumor induction, rats were fed with 100 and 200 mg/kg BW of PFPE. **a** Tumor volumes were recorded during the treatment period. **b** Tumors were isolated and weighted at the end of the study. **c** Representative images of the *in vivo* animal imaging system of each group of rats using IntegriSense 680. The fluorescence signals indicated the location of the cancer cells. **d** The formed tumor was quantified by measuring radiant efficiency. Data are presented as the mean ± SEM (n = 5). *P < 0.05 compared with the control group using one-way ANOVA.

anticancer-related compounds, such as piperlonguminine, 2(10)-pinene, *β*-bisabolene, caryophyllene oxide, and kusunokinin, were found to be around 1–1.6% of the peak area. Due to the crystallization step in the PFPE extraction protocol, the percentage of piperine in the dichloromethane extract of *P. nigrum* crude extract decreased from 49.02 to 13.22% (**Table S2**, Supporting Information).

To determine the effect of PFPE on tumor progression, 100 and 200 mg/kg BW of PFPE were used to treat NMU-induced mammary tumor rats 3 times per week for 4 weeks. The results showed that the size of the tumors in the PFPE-treated groups exhibited an obvious difference from the control group starting on day 12 after the first administration (▶ **Fig. 1 a**). The size of the tumors of the control group progressed aggressively until 1700–1800 mm^3 on day 28, while the growth of the tumors in the PFPE groups was in a steady state, with a range of volume between 40–100 mm^3 (▶ **Fig. 1 a**). Additionally, the average tumor weight of the PFPE groups was significantly lower than the control group, especially at 100 mg/kg BW of PFPE (▶ **Fig. 1 b**). To confirm the action of PFPE on the reduction of tumors in rats, *in vivo* imaging was used to track the tumor area in the representative rats using IntegriSense 680 on days 10 and 24 after treatment (▶ **Fig. 1 c**). The tumors in the PFPE treatments formed apparently smaller tumors than the control group. At day 24 after treatment, the average radiant efficiencies of the tumors at 100 and 200 mg/kg BW of the PFPE-treated groups were 7.49×10^8 and 8.63×10^8 [p/s/cm^2/sr]/[μW/cm^2], respectively, which were lower than the control group by approximately 1.6–1.8 times (14.1×10^8 [p/s/cm^2/sr]/[μW/cm^2]) (▶ **Fig. 1 d**). Therefore, the results indicated that PFPE significantly suppressed mammary tumor progression, while a rapid growth of tumors in the control group was observed.

To investigate whether PFPE changed the immune background before the NMU injection and after PFPE treatments, cytokine array analysis was performed. The result showed that seven cytokines appeared on the arrays (▶ **Fig. 2 a, b**). Following intensity analysis of each spot, the signals from all experimental groups were compared to the normal group and displayed as the fold change responding to the normal level. Most of the cytokines before NMU injection demonstrated a slight difference between each group with about ± 0.2-fold changes on the day before NMU, representing the same cytokine background of each group of rats. However, at the end of the experiment, plasma from tumor-bearing rats with or without PFPE feeding showed some cytokine responses with different levels, around ± 0.2- to 0.6-fold changes compared to the normal group (▶ **Fig. 2 c–i**). Cytokine levels before and after treatments within groups with a noticeable pattern was observed. The regular trend of before and after treatments in all groups was demonstrated in CCL5, TIMP-1, CXCL-5, and VEGF with a small downregulation on the sacrifice day, especially in the 200 mg/kg BW PFPE treatment group. Interestingly, CXCL7 and sICAM-1 levels of PFPE groups were decreased on the sacrifice day. Meanwhile, rats in the control group showed an in-

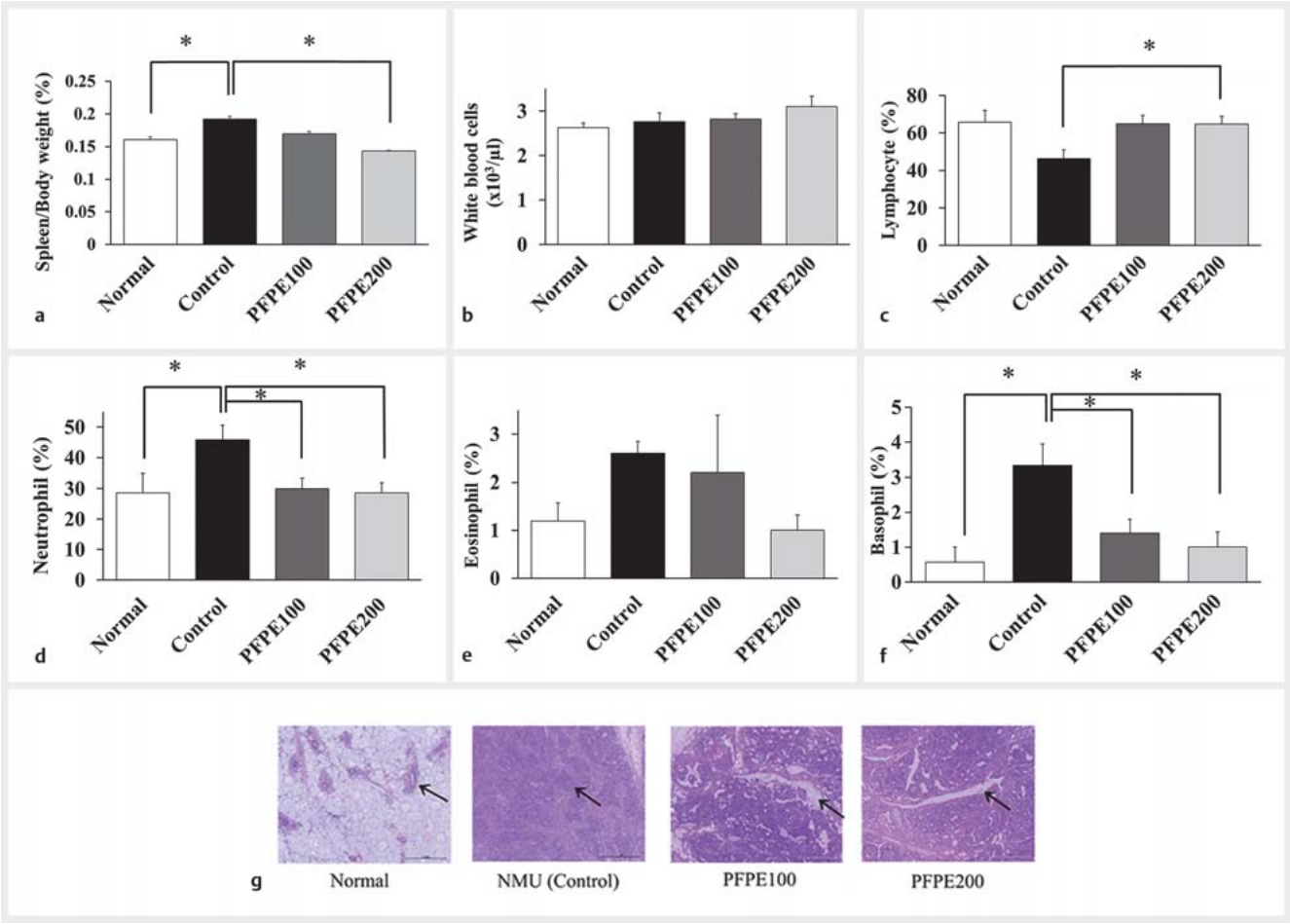


▶ **Fig. 2** Effect of PFPE on the level of cytokines and chemokines. The plasma of rats treated with 100 and 200 mg/kg BW of PFPE was collected. The cytokines and chemokines were determined using a Proteome Profiler Rat Cytokine Array Kit. **a, b** Array images showing spots of each cytokine or chemokine before NMU injection and on the sacrifice day. The ratio of pixel densities in the experimental groups is presented as fold change compared to the normal group at the same period of blood collection. The three duplicated spots without numbers refer to the positive reference spot. Graphs represent (**c**) CCL5, (**d**) CXCL7, (**e**) TIMP-1, (**f**) CXCL5, (**g**) sICAM-1, (**h**) L-selectin, and (**i**) VEGF. The quantitative analysis of the pixel intensity data did not provide p values to demonstrate significance levels.

creased signal, which was around 0.5-fold higher than the plasma obtained on the day before tumor induction. Although the similar expression level on the day before NMU and the day of sacrifice of L-selectin was found in the control group, PFPE treatment down-regulated this cytokine with a factor of 0.2–0.4, especially in the 200 mg/kg BW PFPE treatment group (▶ **Fig. 2h**). These results suggest that PFPE influenced the response of immune components in mammary tumor-bearing rats.

In order to determine the influence of PFPE on systemic immune response, leukocytes and the spleen/BW ratio were determined on the sacrifice day. The number of certain types of circulating leukocytes was counted by flow cytometry. The results showed that the inflammatory response was detected in the control group by a significant increase of the spleen/BW ratio compared to the normal group. Meanwhile, treatment with 200 mg/kg BW of PFPE showed a significantly decreased spleen/BW ratio. In addition, there was no statistically significant difference between normal and PFPE treatments (▶ **Fig. 3a**). Total white blood cells slightly increased in the 200 mg/kg BW PFPE treatment group compared to normal and control groups (▶ **Fig. 3b**). Interestingly, PFPE showed the capability to boost the population of lymphocytes and significantly suppressed the number of neutrophils and basophils compared to the control group (▶ **Fig. 3c, d, f**).

For the H&E staining results of tumor tissues, we found that the control group had a tight density of cancer cells while normal rats had only two layers of cells arranged around the milk ducts. PFPE treatments showed less compaction of cancer cells than the control group. Cancer immune responses were observed in tumor tissues of both the 100 and 200 mg/kg BW PFPE treatment groups (▶ **Fig. 3g**).



▶ **Fig. 3** Effect of PFPE on spleen, BW, circulating leukocytes, and tumor infiltrating cells in NMU-induced tumorigenesis in rats. On the sacrifice day, rats were operated, and organs and leukocytes were collected including (**a**) spleen, (**b**) white blood cells, (**c**) lymphocyte, (**d**) neutrophils, (**e**) eosinophils, and (**f**) basophils. (**g**) Tumors were collected and stained with hematoxylin and eosin (magnification × 100). The normal breast tissue represents milk ducts and adipose tissue (arrow). The large and dense cancer cells represent the control (NMU) group. PFPE at 100 and 200 mg/kg BW caused widespread inflammation and necrosis in the tumor tissue (arrow). Data are presented as the mean ± SEM (n = 5). *P < 0.05 compared with the normal or control group using one-way ANOVA.

In this study, we also determine the side effects of PFPE in rats on the 28th day after treatment. The result showed that there were no significant differences in hematologic and clinical chemistry values of the PFPE-treated rats when compared with the control group, as shown in ▶ **Table 2**. These data indicated the regulatory effects of PFPE on immunohomeostasis in tumor-bearing rats since PFPE restored the number of many leukocytes into the normal range.

To confirm the action of PFPE on the promotion of lymphocytes in tumor-bearing rats, in this experiment, we determined the changes of helper T lymphocyte subtypes, which are related to anticancer immunity. The subpopulations of helper T lymphocyte, including tumor suppressing type 1 T helper cells (Th1; $CD4^+IFN\text{-}\gamma^+$) and tumor-promoting type 2 T helper cells (Th2; $CD4^+IL\text{-}4^+$), were then investigated using flow cytometry. The results showed that rats with tumor progression in the control group represented a slight decrease in Th1 cells when compared to the normal group. However, Th1 cells were significantly increased in the 200 mg/kg BW PFPE treatment group (▶ **Fig. 4a, b**). However, the increasing of the Th2 population was observed in the control group compared to the normal group, while this cell population was reduced to a normal level like the normal group when feeding rats with 100 and 200 mg/kg BW of PFPE (▶ **Fig. 4a, c**). These results indicate that PFPE was able to significantly reduce the amount of Th2 while promoting the number of Th1 cells compared to the control group (▶ **Fig. 4**).

$CD4^+CD25^+$ regulatory T cells ($CD4^+CD25^+$ Treg) are a subpopulation of T lymphocytes functioning as a negative regulator for immune responses. In this study, we found that the number of Treg cells in rats with tumor progression in the control group was significantly higher than the normal rats. However, PFPE treatments at doses 100 and 200 mg/kg BW statistically suppressed Treg cells to a normal level, the same as the normal group (▶ **Fig. 5a, b**). These data indicate that PFPE promoted antitumor immune response by influencing the number of Treg cells.

▶ **Table 2** Hematologic and clinical blood chemistry values of the treatment study on the induced mammary tumorigenesis rats.

Parameters	Normal	Control	PFPE (mg/kg BW)	
			100	200
Hematologic values				
Red blood cells (× 10^6/μL)	7.98 ± 0.48	6.94 ± 0.26	6.96 ± 0.51	7.07 ± 0.30
Hemoglobin (g/dL)	14.91 ± 0.61	14.25 ± 0.23	13.53 ± 0.81	14.34 ± 0.38
Hematocrit (%)	43.14 ± 2.61	39.50 ± 0.81	38.17 ± 2.44	38.00 ± 1.38
Mean corpuscular volume (fL)	55.00 ± 0.65	56.10 ± 0.60	52.83 ± 0.79	54.00 ± 0.62
Mean corpuscular hemoglobin (pg)	18.99 ± 0.16	20.72 ± 0.61	19.60 ± 0.43	20.44 ± 0.35
Mean corpuscular hemoglobin concentration (g/dL)	34.57 ± 0.53	37.01 ± 1.01	37.02 ± 0.75	37.90 ± 0.77
Platelet (× 10^5/μL)	5.78 ± 0.60	7.26 ± 0.45	7.46 ± 0.74	6.96 ± 0.82
Clinical blood chemistry values				
BUN (mg/dL)	26.31 ± 1.45	24.33 ± 1.28	22.77 ± 2.06	24.82 ± 1.85
Creatinine (mg/dL)	0.50 ± 0.04	0.51 ± 0.03	0.54 ± 0.06	0.45 ± 0.02
Albumin (g/dL)	4.26 ± 0.20	4.13 ± 0.08	4.22 ± 0.08	4.07 ± 0.11
SGOT (U/L)	133. 86 ± 29.16	98.50 ± 10.99[a]	97.00 ± 8.31[a]	108.89 ± 12.31[a]
SGPT (U/L)	54.71 ± 1 8.83	39.20 ± 2.56[a]	38.16 ± 2.27[a]	40.56 ± 1.91[a]
Alkaline phosphatase (U/L)	84.00 ± 8.48	78.10 ± 6.90	79.67 ± 5.96	72.78 ± 7.12

Values represent the mean ± SEM of five rats. [a] $P < 0.05$ significantly difference compared with the normal group using one-way ANOVA analysis. SGOT = serum glutamic oxaloacetic transaminase, SGPT = serum glutamic pyruvic transaminase, BUN = blood urea nitrogen

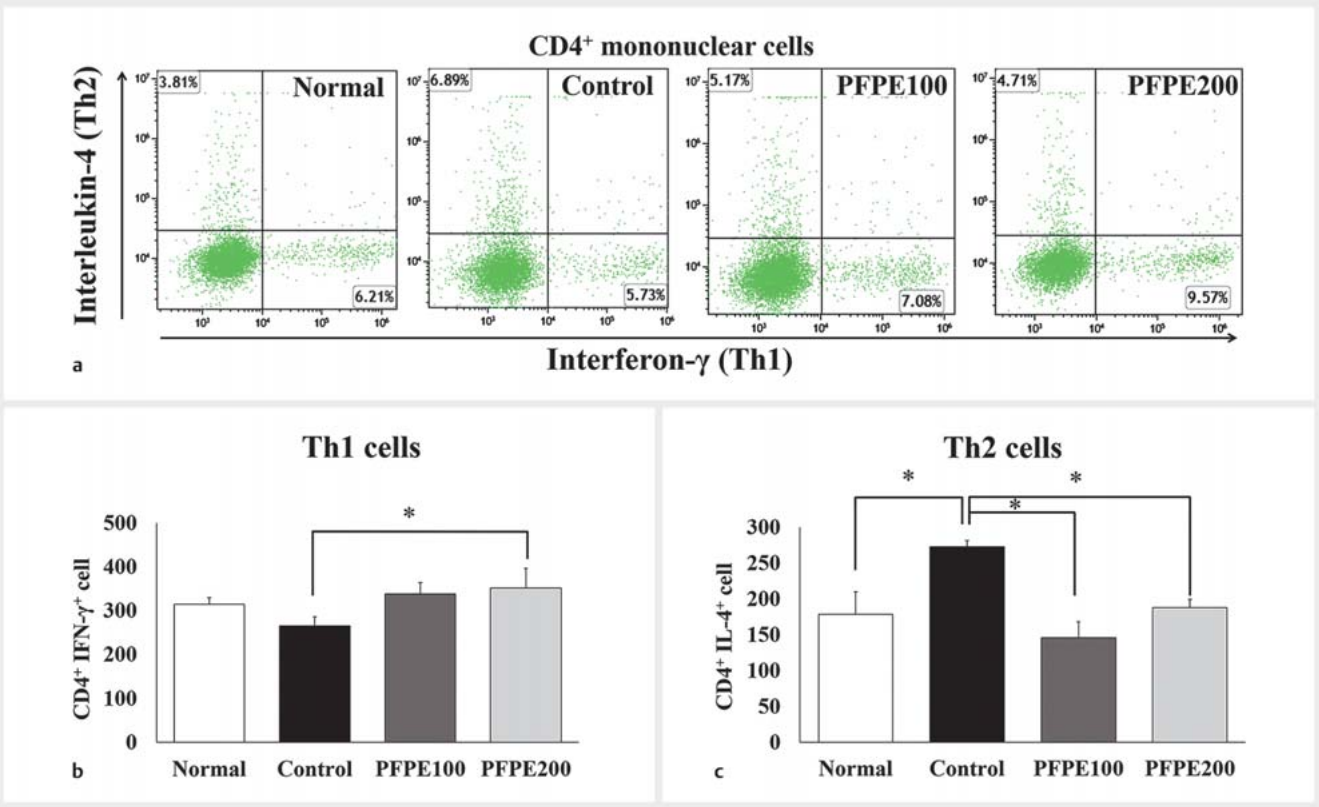
Discussion

P. nigrum is one spice that has exhibited pharmacological properties against many diseases, including infections, diarrhea, fever, and inflammation [3]. Natural chemical components from *P. nigrum* have been well documented. For example, *β*-caryophyllene has been demonstrated to contain anticancer and antioxidant properties [21]. Moreover, pellitorine showed the ability to inhibit cancer cell survival [35]. Another compound, *α*-humulene, also suppressed the production of inflammatory cytokines, TNF-*α*, and IL-1*β* in rats [26]. These all suggest the anti-inflammatory, antioxidant, and anticancer abilities of *P. Nigrum*-derived compounds. Previously, we reported that PFPE, a crude extract of *P. nigrum* with a low amount of piperine, contains six chemical groups, including alkaloids, terpenes, amides, lignans, opioids and steroids. The highest percentages of peak areas with antioxidant, anti-inflammation, or anticancer properties were 13.28% for caryophyllene (terpene) and 1.28% for kusunokinin (lignan). In this study, our work showed a similar result on the components of PFPE with previous work (**Fig. S1,** Supporting Information) [19].

We also evaluated whether PFPE caused side effects. PFPE showed no significant difference in hematologic and clinical chemistry parameters when rats were treated with PFPE. However, we noticed a significant decrease of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) values of the control and PFPE treatment groups, which was significantly lower than normal rats. These effects could be due to the action of NMU [36]. However, PFPE at a dose of 200 mg/kg BW tended to improve both values.

Our previous research has shown that PFPE inhibited breast cancer cells at doses of 100 and 200 mg/kg BW and increased reactive oxygen species [5, 20]. PFPE inhibited breast cancer cells by inhibiting cell proliferation and migration proteins, including E-cadherin, c-myc, VEGF, MMP-9, and MMP-2 [5].

Nowadays, it is well documented that antitumor immunity is a complex network consisting of a wide range of immune effector cells and cytokine interaction. The result from the cytokine array demonstrated that PFPE showed an interaction with cytokine production, particularly the suppression of pro-tumorigenic cytokine CXCL7, which has been reported to be associated with many aspects of tumor progression [37]. Interestingly, 3-carene and pellitorine found in PFPE have been reported to inhibit the production of CXCL7 mediated by the suppression of the activator IL-6, leading to tumor regression [22]. Moreover, CXCL7 plays an important role in neutrophil attraction and activation in the inflammatory response [38]. This may explain our results showing the lower number of circulating neutrophils in PFPE-treated rats while higher levels of both neutrophils and CXCL7 were found in the control group. In addition to CXCL7, the pro-tumorigenic cytokine sICAM-1 was also affected by PFPE. sICAM-1 is a soluble form of ICAM-1, which is found in a high concentration in several kinds of cancers [39]. Many studies have shown the competitive role of sICAM-1 with membrane-bound ICAM-1, which may inhibit immune recognition of tumor cells [40]. Interestingly, caryophyllene and *α*-humulene can suppress sICAM-1 production [41],

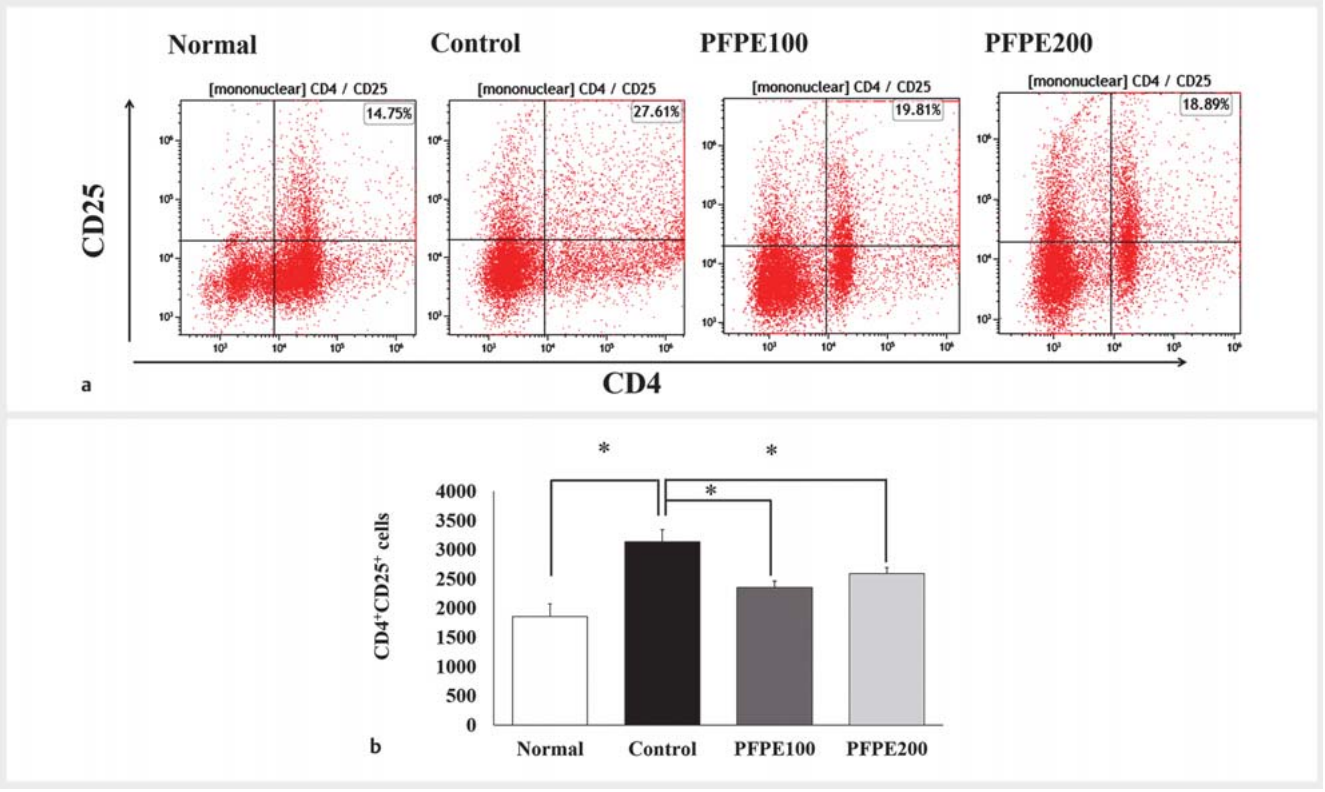


▶ **Fig. 4** Flow cytometry analysis of circulating Th1 and Th2 in NMU-induced mammary tumor rats treated with 100 and 200 mg/kg BW of PFPE. **a** The representative dot plot of Th1 ($CD4^+IFN\text{-}\gamma^+$) and Th2 ($CD4^+IL\text{-}4^+$) proportions of each group of rats are demonstrated. **b** The number of Th1 cells per 5000 $CD4^+$ cells. **c** The number of Th2 cells per 5000 $CD4^+$ cells. Data are presented as the mean ± SEM (n = 5). $^*P < 0.05$ compared with the normal or control group using one-way ANOVA.

which may explain how PFPE suppressed the level of sICAM-1 in tumor-bearing rats. According to all of the evidence as we recently described, the anti-inflammation and antioxidant properties of PFPE could be the main functional activity that helped to promote an antitumor immune response.

In addition to the cytokine response study, we examined the impact of PFPE on systemic immune responses, including the spleen/BW ratio, circulating blood neutrophils, basophils, eosinophils, and number of helper T lymphocyte subtypes (Th1, Th2, and Treg). Interestingly, rats receiving NMU alone seem to show systemic inflammation since a higher spleen/BW ratio and neutrophils were observed compared to other groups. In fact, neutrophil activation has commonly been reported to be associated with systemic inflammation and tumor promotion induced by neutrophil extracellular traps occurring in the tumor microenvironment [42]. This is in accordance with our H&E staining of tumor tissue showing the infiltration of the immune cells around the tumor tissue. Although this infiltration also occurred in the PFPE groups, the spleen/BW ratio and circulating neutrophils were decreased in PFPE feeding rats, indicating a weaker inflammatory response. This anti-inflammation effect of PFPE could be due to the suppression of the neutrophil activator CXCL-7, as shown in the cytokine array analysis.

In addition, we also evaluated the specific types of T cells; Th1 cells, which have been reported for their antitumor activity, and Th2 cells, tumor-promoting T cells. Our flow cytometry showed that feeding tumor-bearing rats with 200 mg/kg BW of PFPE significantly increased the number of Th1 cells compared to other groups of rats. Moreover, this dose of PFPE suppressed the proportion of Th2 cells into the normal range. Importantly, this suggested that 200 mg/kg BW of PFPE was optimal for induction of antitumor immunity since the Th1 promotion and Th2 suppression activity of PFPE were demonstrated at this dose, which has not been reported before. Moreover, although without directed study, caryophyllene, a major component of PFPE, seems to influence the Th1/Th2 balance by activating IFN-γ production, which is a Th1-promoting cytokine [43]. Treg, one of the targets for cancer immunotherapy, contains immune suppressing activity that inhibits anticancer immunity [44]. Many immune checkpoint inhibitors and other strategies have been developed to suppress this type of immune cell [44]. Importantly, PFPE also significantly decreased the number of Treg cells, immune suppressing cells, compared to the control group, therefore, this result emphasized the immune boosting activity of PFPE. Taken together, we concluded that, in addition to the directed killing activity to cancer cells, PFPE promoted the antitumor immune response via regulating the Th1/Th2 balance and Treg suppression. Therefore, further studies



▶ **Fig. 5** Flow cytometry analysis of circulating Treg in NMU-induced mammary tumor rats treated with 100 and 200 mg/kg BW of PFPE. **a** The representative dot plot of Treg ($CD4^+CD25^+$) proportions of each group of rats are demonstrated. **b** The number of Treg cells per 10 000 mononuclear cells. Data are presented as the mean ± SEM (n = 5). $^*P < 0.05$ compared with the normal or control group using one-way ANOVA.

should investigate the effect of PFPE on other immune cells related to the tumor microenvironment, such as myeloid-derived suppressor cells, N1/N2 neutrophils, M1/M2 macrophages, or cancer-associated fibroblast cells.

In conclusion, our findings demonstrated the anticancer immunity of PFPE in the suppression of breast tumor growth in rats. This crude extract contained many antioxidant, anti-inflammation, and anticancer compounds. PFPE obviously decreased tumor volume without side effects on blood biochemical parameters of treated rats. According to flow cytometry and cytokine array results, the optimal dose of PFPE was 200 mg/kg BW, which was the most effective concentration that promoted Th1, suppressed Th2 and Treg, and inhibited the expression of CXCL7. PFPE also displayed anti-inflammatory properties, especially the inhibition of neutrophils and basophils and the reduction of cytokines and chemokines related to immune recognition inhibition. Taken together, these data indicate that PFPE can be used as a promising crude extract for immunostimulants for breast cancer treatment.

Methods

Plant materials and crude extract

Dried fruits of black peppercorn were collected from Nakhon Si Thammarat province with less than 13% moisture content. PFPE extract was prepared by modifying Reshmi's method as previously described [20]. Quantitative analysis of the composition of PFPE was carried out using GC-MS according to a method described previously [18].

Animal model and assessment of treatment effect

Female Sprague-Dawley rats (150–180 g BW), 45 days of age, were obtained from Nomura Siam International Co., Ltd. and maintained in the Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University. All animal procedures were conducted under the guidelines approved by the Animal Care and Use Committee of Prince of Songkla University (animal experimentation application Ref. 44/2019; date of approval: June 19, 2019). The housing conditions were followed: a temperature of 22 ± 3 °C with a relative humidity of 30 ± 10%, free access to clean food and water, and a 12-h light-dark cycle.

Rats were randomly separated into 4 groups of 10 animals each. Group 1 (normal control) received no treatment and no NMU. Groups 2 to 4 were injected intraperitoneally with 250 mg/kg BW of NMU to induce breast cancer at 50 and 80 days of age. After 7 days of the last NMU injection, rats were fed with 100 µg/kg BW of estradiol for 10 days. The experimental treatment was begun after a tumor diameter reached 0.2–0.5 cm, and the day the treatment started was defined as day 0. Group 2 (tumor control) was fed with medium chain triglycerides and 2% vitamin E.

Groups 3 and 4 were fed with PFPE at 100 and 200 mg/kg BW, respectively, 3 times per week for 4 weeks. Tumor volumes were measured by caliper 3 times a week, and tumor volume was calculated by using the equation $V = 0.4\,ab^2$, where "a" and "b" represent the largest tumor diameter and the next largest tumor diameter, respectively. After 4 weeks of treatment, the rats were sacrificed, and the blood was collected for flow cytometry and hematologic and biochemical analysis. Tumors were isolated for weight and size analysis.

In vivo imaging

On days 10 and 24 after the first treatment, a representative rat from each group was anesthetized and intravenously injected with 12 nmol/600 µL of IntegriSense 680 (Perkin Elmer). Twenty-four hours after injection, the tumor was measured using an IVIS Lumina III system (Perkin Elmer) and regions of interest were designed around each mammary tumor. Data are expressed as average radiant efficiency by Living Image software (supplied with an IVIS Lumina III system).

Histopathologic study

The mammary tumor tissues were isolated and fixed in 10% buffered formalin solution. Then, tumor tissues were embedded in paraffin using a conventional automated system and stained with H&E for histopathologic examination. The paraffin sections of each tissue image were captured by light microscopy [20].

Mononuclear cells isolation

Rats were deeply anesthetized by injecting 200 mg/kg BW of pentobarbital (Sigma-Aldrich) intraperitoneally, and blood samples were extracted by cardiac puncture. The blood was mixed with serum-free RPMI-1640 medium (Gibco) at a 1:1 ratio and then overlaid on Lymphoprep (Stemcell Technologies) gradient medium (1.077 g/mL density) in a 15-mL centrifuge tube. The filled Lymphoprep tube was then centrifuged for 30 min at 800 *g* with the lowest descending and ascending rates. The peripheral blood mononuclear cells were collected from the interphase between the plasma and Lymphoprep layers.

Flow cytometry

The isolated mononuclear cells were counted by a hemacytometer and incubated with a cell activation cocktail (BD Biosciences) containing complete RPMI medium at 2×10^6 cells/mL for 4 h at 37 °C in a CO_2 incubator. Cells were then washed and stained with FITC mouse anti-rat CD4 and/or PE mouse anti-rat CD25 antibodies (BD Biosciences). After incubation on ice for 30 min in a dark environment, cell surface-stained mononuclear cells were then fixed with a fixation buffer (BD Biosciences) and washed 2 times with permeabilization wash buffer (BD Biosciences). In the staining step, cells were incubated with antibodies against intracellular cytokines (Alex 647 mouse anti-rat IFN-γ and PE mouse anti-rat IL-4 antibodies) at 4 °C for 30 min in complete darkness. The stained cells were then washed and resuspended in cell staining buffer before flow cytometry analysis using the Beckman CytoFLEX S platform (Beckman Coulter), and data were analyzed using Kaluza analysis flow cytometry software (Beckman Coulter)

Cytokine array assay

A rat cytokine antibody array was conducted using a Proteome Profiler Rat Cytokine Array Kit, Panel A (R&D Systems). The kit consisted of an antibody spotted membrane that detects 29 different cytokines/chemokines in one experiment. The protocol was conducted according to the manufacturer's instructions. The detection was performed by exposure to a peroxidase substrate, and the signal was captured by using Alliance Q9 (Uvitec). Cytokine array TIF images were then used to analyze the intensity score using UVITEC Alliance software (Uvitec) from both signals. The data were expressed as the relative fold change compared with the normal group.

Statistical analysis

Animal data were analyzed by one-way ANOVA. All data are presented as mean ± SEM. Multiple conditions were compared using a parametric one-way ANOVA using SPSS, version 24.0 (The International Business Machines Corporation). A p value of < 0.05 was statistically significant.

Supporting information

Contents of compound and chromatogram pattern of PFPE analyzed by GM-MS are available as Supporting Information.

Contributors' Statement

Conception and design of the work: J. Saetang, S. Sangkhathat, A. Tedasen, N. Sangkaew, S. Dokduang, N. Prompat, S. Taraporn, P. Graidist; data collection: J. Saetang, A. Tedasen, N. Sangkaew, S. Dokduang, N. Prompat, S. Taraporn; analysis and interpretation of the data: J. Saetang, A. Tedasen, S. Sangkhathat, N. Sangkaew, S. Dokduang, N. Prompat, S. Taraporn, P. Graidist; statistical analysis: J. Saetang, A. Tedasen, S. Sangkhathat, N. Sangkaew, N. Prompat, S. Dokduang, S. Taraporn; drafting the manuscript: J. Saetang, S. Sangkhathat, P. Graidist; critical revision of the manuscript: J. Saetang, P. Graidist.

Acknowledgements

This research project was financially supported by the Agricultural Research Development Agency (Public Organization) (CRP6205031530), Thailand and the Postdoctoral Fellowship Program, Faculty of Medicine, Prince of Songkla University. We would like to acknowledge the Central Research Laboratory, Faculty of Medicine, Prince of Songkla University for the use of all laboratory facilities. We also thank the Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University for animal care and special consults.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Chatterjee S, Niaz Z, Gautam S, Adhikari S, Variyar P, Sharma A. Antioxidant activity of some phenolic constituents from green pepper (*Piper nigrum* L.) and fresh nutmeg mace (*Myristica fragrans*). Food Chem 2007; 101: 515–523

[2] Morsy NFS, El-Salam EAA. Antimicrobial and antiproliferative activities of black pepper (*Piper nigrum* L.) essential oil and oleoresin. J Essent Oil Bear Plants 2017; 20: 779–790

[3] Salehi B, Zakaria ZA, Gyawali R, Ibrahim SA, Rajkovic J, Shinwari ZK, Khan T, Sharifi-Rad J, Ozleyen A, Turkdonmez E, Valussi M, Tumer TB, Monzote Fidalgo L, Martorell M, Setzer WN. *Piper* species: A comprehensive review on their phytochemistry, biological activities and applications. Molecules 2019; 24: 1364

[4] Butt MS, Pasha I, Sultan MT, Randhawa MA, Saeed F, Ahmed W. Black pepper and health claims: a comprehensive treatise. Crit Rev Food Sci Nutr 2013; 53: 875–886

[5] Deng Y, Sriwiriyajan S, Tedasen A, Hiransai P, Graidist P. Anti-cancer effects of *Piper nigrum* via inducing multiple molecular signaling *in vivo* and *in vitro*. J Ethnopharmacol 2016; 188: 87–95

[6] Prashant A, Rangaswamy C, Yadav AK, Reddy V, Sowmya MN, Madhunapantula S. *In vitro* anticancer activity of ethanolic extracts of *Piper nigrum* against colorectal carcinoma cell lines. Int J Appl Basic Med Res 2017; 7: 67–72

[7] Xie Z, Wei Y, Xu J, Lei J, Yu J. Alkaloids from *Piper nigrum* synergistically enhanced the effect of paclitaxel against paclitaxel-resistant cervical cancer cells through the downregulation of Mcl-1. J Agric Food Chem 2019; 67: 5159–5168

[8] Tasleem F, Azhar I, Ali SN, Perveen S, Mahmood ZA. Analgesic and anti-inflammatory activities of *Piper nigrum* L. Asian Pac J Trop Med 2014; 7S1: S461–S468

[9] D'cruz SC, Mathur PP. Effect of piperine on the epididymis of adult male rats. Asian J Androl 2005; 7: 363–368

[10] Allameh A, Saxena M, Biswas G, Raj HG, Singh J, Srivastava N. Piperine, a plant alkaloid of the piper species, enhances the bioavailability of aflatoxin B1 in rat tissues. Cancer Lett 1992; 61: 195–199

[11] Teng MWL, Galon J, Fridman WH, Smyth MJ. From mice to humans: developments in cancer immunoediting. J Clin Invest 2015; 125: 3338–3346

[12] Zou JY, Su CH, Luo HH, Lei YY, Zeng B, Zhu HS, Chen ZG. Curcumin converts Foxp3+ regulatory T cells to T helper 1 cells in patients with lung cancer. J Cell Biochem 2018; 119: 1420–1428

[13] Dandawate S, Williams L, Joshee N, Rimando AM, Mittal S, Thakur A, Lum LG, Parajuli P. *Scutellaria* extract and wogonin inhibit tumor-mediated induction of T(reg) cells via inhibition of TGF-β1 activity. Cancer Immunol Immunother 2012; 61: 701–711

[14] Katiyar SK, Challa A, McCormick TS, Cooper KD, Mukhtar H. Prevention of UVB-induced immunosuppression in mice by the green tea polyphenol (−)-epigallocatechin-3-gallate may be associated with alterations in IL-10 and IL-12 production. Carcinogenesis 1999; 20: 2117–2124

[15] Bui TT, Piao CH, Hyeon E, Fan Y, Van Nguyen T, Jung SY, Choi DW, Lee SY, Shin HS, Song CH, Chai OH. The protective role of *Piper nigrum* fruit extract in an ovalbumin-induced allergic rhinitis by targeting of NFκBp65 and STAT3 signalings. Biomed Pharmacother 2019; 109: 1915–1923

[16] Bui TT, Piao CH, Song CH, Shin HS, Shon DH, Chai OH. *Piper nigrum* extract ameliorated allergic inflammation through inhibiting Th2/Th17 responses and mast cells activation. Cell Immunol 2017; 322: 64–73

[17] Majdalawieh AF, Carr RI. *In vitro* investigation of the potential immunomodulatory and anti-cancer activities of black pepper (*Piper nigrum*) and cardamom (*Elettaria cardamomum*). J Med Food 2010; 13: 371–381

[18] Tedasen A, Dokduang S, Sukpondma Y, Lailerd N, Madla S, Sriwiriyajan S, Rattanaburee T, Tipmanee V, Graidist P. (−)-Kusunokinin inhibits breast cancer in N-nitrosomethylurea-induced mammary tumor rats. Eur J Pharmacol 2020; 882: 173311

[19] Tedasen A, Khoka A, Madla S, Sriwiriyajan S, Graidist P. Anticancer effects of piperine-free *Piper nigrum* extract on cholangiocarcinoma cell lines. Pharmacogn Mag 2020; 16: 28

[20] Sriwiriyajan S, Tedasen A, Lailerd N, Boonyaphiphat P, Nitiruangjarat A, Deng Y, Graidist P. Anticancer and cancer prevention effects of piperine-free *Piper nigrum* extract on N-nitrosomethylurea-induced mammary tumorigenesis in rats. Cancer Prev Res (Phila) 2016; 9: 74–82

[21] Dahham SS, Tabana YM, Iqbal MA, Ahamed MB, Ezzat MO, Majid AS, Majid AM. The Anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of *Aquilaria crassna*. Molecules 2015; 20: 11808–11829

[22] Basholli-Salihu M, Schuster R, Hajdari A, Mulla D, Viernstein H, Mustafa B, Mueller M. Phytochemical composition, anti-inflammatory activity and cytotoxic effects of essential oils from three *Pinus* spp. Pharm Biol 2017; 55: 1553–1560

[23] Ngo QMT, Tran PT, Tran MH, Kim JA, Rho SS, Lim CH, Kim JC, Woo MH, Choi JS, Lee JH, Min BS. Alkaloids from *Piper nigrum* exhibit antiinflammatory activity via activating the Nrf2/HO-1 pathway. Phytother Res 2017; 31: 663–670

[24] Chandra M, Prakash O, Kumar R, Bachheti RK, Bhushan B, Kumar M, Pant AK. β-selinene-rich essential oils from the parts of *Callicarpa macrophylla* and their antioxidant and pharmacological activities. Medicines (Basel) 2017; 4: 52

[25] Chen JJ, Huang YC, Chen YC, Huang YT, Wang SW, Peng CY, Teng CM, Chen IS. Cytotoxic amides from *Piper sintenense*. Planta Med 2002; 68: 980–985

[26] Fernandes ES, Passos GF, Medeiros R, da Cunha FM, Ferreira J, Campos MM, Pianowski LF, Calixto JB. Anti-inflammatory effects of compounds alpha-humulene and (−)-trans-caryophyllene isolated from the essential oil of *Cordia verbenacea*. Eur J Pharmacol 2007; 569: 228–236

[27] Reynoso-Moreno I, Najar-Guerrero I, Escareño N, Flores-Soto ME, Gertsch J, Viveros-Paredes JM. An endocannabinoid uptake inhibitor from black pepper exerts pronounced anti-inflammatory effects in mice. J Agric Food Chem 2017; 65: 9435–9442

[28] Sriwiriyajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. Cytotoxicity screening of plants of genus *Piper* in breast cancer cell lines. Trop J Pharm Res 2014; 13: 921

[29] Ma C, Zhou W, Yan Z, Qu M, Bu X. β-Elemene treatment of glioblastoma: a single-center retrospective study. Onco Targets Ther 2016; 9: 7521–7526

[30] Salehi B, Upadhyay S, Erdogan Orhan I, Kumar Jugran A, L D Jayaweera S, A Dias D, Sharopov F, Taheri Y, Martins N, Baghalpour N, Cho WC, Sharifi-Rad J. Therapeutic potential of α- and β-pinene: a miracle gift of nature. Biomolecules 2019; 9: 738

[31] Yeo SK, Ali AY, Hayward OA, Turnham D, Jackson T, Bowen ID, Clarkson R. β-bisabolene, a sesquiterpene from the essential oil extract of opoponax (*Commiphora guidottii*), exhibits cytotoxicity in breast cancer cell lines. Phytother Res 2016; 30: 418–425

[32] Rattanaburee T, Thongpanchang T, Wongma K, Tedasen A, Sukpondma Y, Graidist P. Anticancer activity of synthetic (±)-kusunokinin and its derivative (±)-bursehernin on human cancer cell lines. Biomed Pharmacother 2019; 117: 109115

[33] de Souza Grinevicius VMA, Kviecinski MR, Santos Mota NSR, Ourique F, Porfirio Will Castro LS, Andreguetti RR, Gomes Correia JF, Filho DW, Pich CT, Pedrosa RC. *Piper nigrum* ethanolic extract rich in piperamides causes ROS overproduction, oxidative damage in DNA leading to cell cycle arrest and apoptosis in cancer cells. J Ethnopharmacol 2016; 189: 139–147

[34] Guan C, Liu W, Yue Y, Jin H, Wang X, Wang XJ. Inhibitory effect of β-elemene on human breast cancer cells. Int J Clin Exp Pathol 2014; 7: 3948–3956

[35] Ee GC, Lim CM, Rahmani M, Shaari K, Bong CF. Pellitorine, a potential anti-cancer lead compound against HL60 and MCT-7 cell lines and microbial transformation of piperine from *Piper nigrum*. Molecules 2010; 15: 2398–2404

[36] Chang YC, Hsu JD, Lin WL, Lee YJ, Wang CJ. High incidence of acute promyelocytic leukemia specifically induced by N-nitroso-N-methylurea (NMU) in Sprague-Dawley rats. Arch Toxicol 2012; 86: 315–327

[37] Grépin R, Guyot M, Giuliano S, Boncompagni M, Ambrosetti D, Chamorey E, Scoazec JY, Negrier S, Simonnet H, Pagès G. The CXCL7/CXCR1/2 axis is a key driver in the growth of clear cell renal cell carcinoma. Cancer Res 2014; 74: 873–883

[38] von Hundelshausen P, Petersen F, Brandt E. Platelet-derived chemokines in vascular biology. Thromb Haemost 2007; 97: 704–713

[39] Yamada M, Yanaba K, Takehara K, Sato S. Clinical significance of serum levels of soluble intercellular adhesion molecule-1 and soluble L-selectin in malignant melanoma. Arch Dermatol Res 2005; 297: 256–260

[40] Schellerer VS, Langheinrich MC, Zver V, Grützmann R, Stürzl M, Gefeller O, Naschberger E, Merkel S. Soluble intercellular adhesion molecule-1 is a prognostic marker in colorectal carcinoma. Int J Colorectal Dis 2019; 34: 309–317

[41] Tanaka S, Sakata Y, Morimoto K, Tambe Y, Watanabe Y, Honda G, Tabata M, Oshima T, Masuda T, Umezawa T, Shimada M, Nagakura N, Kamisako W, Kashiwada Y, Ikeshiro Y. Influence of natural and synthetic compounds on cell surface expression of cell adhesion molecules, ICAM-1 and VCAM-1. Planta Med 2001; 67: 108–113

[42] Brostjan C, Oehler R. The role of neutrophil death in chronic inflammation and cancer. Cell Death Discov 2020; 6: 1–8

[43] Rogerio AP, Andrade EL, Leite DF, Figueiredo CP, Calixto JB. Preventive and therapeutic anti-inflammatory properties of the sesquiterpene α-humulene in experimental airways allergic inflammation. Br J Pharmacol 2009; 158: 1074–1087

[44] Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? Cancer Sci 2019; 110: 2080–2089

Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Anticancer Effects of Piperine-Free *Piper nigrum* Extract on Cholangiocarcinoma Cell Lines

Aman Tedasen[1,2], Araya Khoka[3], Siribhorn Madla[1], Somchai Sriwiriyajan[3], Potchanapond Graidist[1,2]

[1]Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, [2]The Excellent Research Laboratory of Cancer Molecular Biology, Prince of Songkla University, [3]Department of Pharmacology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand

Submitted: 01-08-2019 Revised: 30-09-2019 Published: 31-03-2020

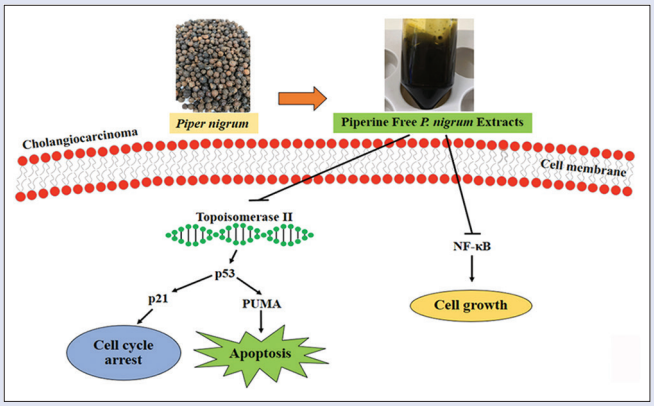
ABSTRACT

Background: Black pepper (*Piper nigrum* L.) is widely used as a traditional medicine, including usage for pain relief, fevers, as well as an anticancer agent. Previously, we reported that piperine-free *P. nigrum* extract (PFPE) inhibited breast cancer *in vitro* and *in vivo*. **Objective:** In this present study, we explored the anticancer effects of PFPE on cholangiocarcinoma (CCA). **Materials and Methods:** 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to analyze cytotoxic potential of PFPE whereas deoxyribonucleic acid (DNA) fragmentation followed by Western blot analysis were used. **Results:** PFPE composed of alkaloid, flavonoid, amide, lignans, opioid, and steroid. This crude extract represented cytotoxic effect against CCA cells which stronger than dichloromethane *P. nigrum* crude extract and piperine, especially on KKU-M213 (median inhibition concentration [IC_{50}] at 13.70 μg/ml) and TFK-1 (IC_{50} at 15.30 μg/ml). Interestingly, PFPE showed lower cytotoxicity against normal human cholangiocyte MMNK-1 cells (IC_{50} at 19.65 μg/ml) than KKU-M213 and TFK-1 cells. Then, the molecular mechanisms of PFPE were firstly evaluated by DNA fragmentation followed by Western blot analysis. The degradation of DNA was observed on KKU-M213 and TFK-1 cells after treatment with PFPE at day 2. Then, proliferation proteins including topoisomerase II, AKT8 virus oncogene cellular homolog, avian myelocytomatosis virus oncogene cellular homolog, cyclin D1, signal transducer and activator of transcription 3, cyclooxygenase-2, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) were decreased and p21 was increased. Furthermore, apoptotic proteins, such as tumor protein p53, Bcl-2-associated X protein, and p53 upregulated modulator of apoptosis were upregulated. Meanwhile, antiapoptotic protein B-cell lymphoma 2 was down-regulated. **Conclusion:** These results indicated that PFPE inhibited CCA through the down-regulation of cell proliferation and induction of apoptosis pathway.

Key words: Anticancer, apoptosis, cell proliferation, cholangiocarcinoma, *Piper nigrum*

SUMMARY

- piperine free *Piper nigrum* extract (PFPE) inhibited cholangiocarcinoma (CCA) cell lines
- PFPE induces CCA cells to undergo apoptosis and cell cycle arrest via the inhibition of topoisomerase II
- PFPE inhibit cell growth through the inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells.



Abbreviations used: PFPE: Piperine free *Piper nigrum* extract; CCA: Cholangiocarcinoma; DPCE: dichloromethane *P. nigrum* crude extract; NMU: N-nitrosomethylurea; ER: Estrogen receptor; MMP-9: Matrix metalloproteinase-9; MMP-2: Matrix metalloproteinase-2; VEGF: Vascular endothelial growth factor; GC-MS: Gas chromatograph-mass spectrometer; MTT: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethylsulfoxide; IC_{50}: Median inhibition concentration; MCLE: Methanol crude extract of *Curcuma longa*; DNA: Deoxyribonucleic acid; STAT-3: Signal transducer and activator of transcription 3; COX-2: Cyclooxygenase-2; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; c-Myc: Avian myelocytomatosis virus oncogene cellular homolog; Akt: AKT8 virus oncogene cellular homolog; Bcl-2: B-cell lymphoma 2; p53: Tumor protein p53; Bax: Bcl-2-associated X protein; PUMA: p53 upregulated modulator of apoptosis.

Correspondence:

Dr. Potchanapond Graidist,
Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand.
E-mail: gpotchan@medicine.psu.ac.th

DOI: 10.4103/pm.pm_288_19

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Cholangiocarcinoma (CCA) is an epithelial cancer originating from the bile ducts with features of cholangiocyte differentiation.[1] There are 2 types of CCA (based on its location) including intrahepatic and extrahepatic.[2] For over the past four decades, incidence of CCA has been increased in United States of America,[3] Australia, England,[4] and Northeastern Thailand.[5] There are several risk factors for CCA, including primary sclerosing cholangitis, liver fluke infections (*Clonorchis sinensis* and *Opisthorchis viverrini*), choledochal cysts, Caroli's disease, hepatitis B and C infection, obesity, cirrhosis and hepatolithiasis.[5,6] The therapeutic for CCA are limited and no current effective treatment because the majority of patients present with advanced stage disease.[7] Even treatments with advances in surgical

Cite this article as: Tedasen A, Khoka A, Madla S, Sriwiriyajan S, Graidist P. Anticancer effects of piperine-free *Piper nigrum* extract on cholangiocarcinoma cell lines. Phcog Mag 2020;16:S28-38.

techniques, chemotherapy and radiotherapy, the 5-year survival rate of patients after diagnosis still remain about 10%.[8] Although surgical resection has improved in the survival of most patients, the recurrent disease was found within 2 years after tumor resection.[9] Chemotherapy and radiation therapy are ineffective and show various side effects such as harmful to normal cells and bone marrow suppression.[10] Therefore, effective therapeutic and alternative treatments with no serious side effect for CCA are urgently needed.

P. nigrum L. belongs to family Piperaceae and can be used as antiapoptotic, antibacterial, anticolon toxin, antidepressant, antifungal, antidiarrhoeal, antiinflammatory, antimutagenic, antimetastatic, antioxidative, antipyretic, antispasmodic, antispermatogenic, antitumor, antithyroid, ciprofloxacin potentiator, cold extremities, gastric ailments, hepatoprotective, insecticidal, intermittent fever, and larvicidal activities.[11] The chemical constituents of *P. nigrum* are aromatic essential oils, alkaloids, amides, prophenylphenols, lignans, terpenes, flavones, and steroids.[12] Ethanolic crude extract of *P. nigrum* consists of high total phenol content shows antioxidant and anti-inflammation as well as cytotoxic property against colorectal carcinoma cell lines.[13] Using ethanol and high pressure (200 bar), *P. nigrum* crude extracts exhibits cytotoxicity against MCF-7 with median inhibition concentration (IC_{50}) of 14.40 ± 3.30 μg/ml and represents tumor inhibitory effect in mammary adenocarcinoma mouse.[14] Previously, we reported that piperine-free *P. nigrum* extract (PFPE) strongly inhibited breast cancer MCF-7 cells with IC_{50} value of 7.45 μg/ml. Moreover, PFPE inhibited tumor growth in *N*-nitrosomethylurea-induced mammary tumorigenesis rats without liver and kidney toxicity.[15] Interestingly, PFPE upregulated tumor protein p53 (p53) and downregulated estrogen receptor, E-cadherin, matrix metalloproteinase-9 (MMP-9), MMP-2, avian myelocytomatosis virus oncogene cellular homolog (c-Myc) and vascular endothelial growth factor (VEGF) *in vitro* and *in vivo*.[16] In this present research, we further explored the phytochemical component, investigated cytotoxicity and molecular mechanisms of PFPE on CCA cell lines.

MATERIALS AND METHODS

Preparation of piperine free *Piper nigrum* extract

Seeds of *P. nigrum* L. were collected from Songkhla province in Thailand. The plant specimen (voucher specimen number SKP 146161401) was identified by Asst. Prof. 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. PFPE was prepared as previously described. Briefly, grounded 250 g of dried seeds of *P. nigrum* L. were soaked in 300 mL of dichloromethane and incubated at 35°C for 3 h in a shaking incubator. After filtration with Whatman filter paper No. 1 and concentration using rotary evaporator, the dark brown oil residue of extracts was obtained and then recrystallized with cold diethyl ether in an ice bath to get rich of yellow crystals (piperine) and obtain brown oil residue (PFPE).[15] PFPE was kept in a desiccator until used.

Phytochemical analysis and identification of bioactive constituents by gas chromatograph-mass spectrometer

The analysis of the phytochemical screening and composition of PFPE extracts were carried out using a Gas Chromatography-Agilent 7890B combination with an Agilent 5977A triple quadrupole mass spectrometer (Agilent Technologies Inc, USA). Gas chromatograph-mass spectrometer (GC-MS) analysis is a common confirmation test, which used to make an effective chemical analysis. The PFPE samples were evaluated phytochemicals such as a flavonoids, tannins, alkaloids, steroids, phenols, glycosides, lignans, and terpenoids. An inlet temperature of 280°C with the split ratio 7:1 was employed and the helium was used as the carried gas at the constant flow rate of 7 ml/min. The oven temperature was initially maintained at 60°C for 5 min and increase at a rate of 5°C/min to 315°C for 15 min. For MS detection, an electron ionization mode was used with an ionization energy of 70 eV, ion source temperature of 230°C, and scan mass range m/z 35–500. The components were identified based on a correlation of the recorded fragmentation patterns of mass spectra that provided in the GC-MS system software version Wiley10 and NIST14. All procedures were performed at Scientific Equipment Center, Prince of Songkla University, Songkhla, Thailand.

Measuring total phenolic, tannin, flavonoid content and radical scavenging activity

The total phenolic content was determined based on Folin–ciocalteu method. Gallic acid was used as the standard and total phenolics were expressed as mg gallic acid equivalent/mg extract (mg GAE/mg extract). Total condensed tannin was measured based on HCL-vanillin method and catechin was used as the standard. The total tannin was reported as mg catechin equivalent/mg extract (mg CE/mg extract). The total flavonoid content was determined by aluminum chloride solution ($AlCl_3$) colorimetric method. Quercetin was employed as the standard and expressed the total flavonoids as mg quercetin equivalent/mg extract (mg QE/mg extract). 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity was performed according to the DPPH trolox assay and reported as mg trolox equivalent antioxidant capacity/mg extract (mg TEAC/mg extract). All procedures were performed at Center of Excellence in Natural Products Innovation, Mae Fah Luang University, Chiang Rai, Thailand.

Cell lines and culture conditions

Three CCA (KKU-100, KKU-M213 and KKU-M055) and one cholangiocyte (MMNK-1) cells were kindly donated by Dr. Mutita Junking (Faculty of Medicine, Mahidol University, Bangkok, Thailand). TFK-1 cells were obtained from RIKEN BioResource Center and HuCC-T1 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank. Mouse fibroblast, L-929 cells, were kindly donated by Associate Professor Dr. Jasadee Kaewsichan (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand).

KKU-100, KKU-M213, KKU-M055, MMNK-1 and L-929 cells were grown in DMEM medium (Invitrogen), which contained 10% of fetal bovine serum (Invitrogen), 2 mmol/L of L-glutamine (Invitrogen), and an antibiotic mixture of 100 units/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen). TFK-1 and HuCC-T1 cells were grown in RPMI 1640 (Invitrogen) supplemented with the same supplement as for DMEM. All cells were maintained by incubating in a 5% CO_2 atmosphere, at 37°C and 96% relative humidity.

In vitro cytotoxicity

The cytotoxicity assay was performed in 96-well plate. KKU-100, KKU-M055, and MMNK-1 cells were seeded at a density of 5×10^3 cells/well. KKU-M213, TFK-1, and HuCC-T1 cells were seeded at a density of 7.5×10^3 cells/well and L-929 cells were seeded at a density of 8×10^3 cells/well. After incubation for 24 h, cells were treated with PFPE at various concentration for 48 h. The cells were then washed with 1X PBS and incubated in 100 μl of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution at 37°C for 30 min. Under light protection, the purple crystals of formazan or MTT metabolites were dissolved with 100 μl of dimethyl

sulfoxide and incubate at 37°C for 30 min. The absorbance was measured at 570 and 650 nm using a microplate reader spectrophotometer (Spectra Max M5, Molecular Devices), and the IC_{50} values were calculated.[17] According to US NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with IC_{50} value ≤20 μg/ml.[18]

Deoxyribonucleic acid fragmentation analysis

KKU-M213 and TFK-1 cells in their exponential growth phase were seeded into 6 cm culture plate at a density of 2.5×10^5 cells/plate for 24 h and then treated with PFPE at 3 folds of IC_{50} values. After treatment for 96 h, cells were harvested by trypsinization. Cell pellets were lysed using the extraction buffer (containing 0.7 M NaCl, 17 mM SDS, 10 mM Tris-HCl (pH 8.0) and 2 mM EDTA (pH 8.0)) and fragmented deoxyribonucleic acid (DNA) in the supernatant was extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1). The DNA was precipitated with a two-thirds volume of cold isopropanol followed by centrifugation at 8,000 ×*g* and washed once in 70% ethanol. Finally, DNA pellet was resuspended in deionized water and analyzed by 1.5% agarose gel electrophoresis.[19]

Western blot analysis

KKU-M213 and TFK-1 cells were seeded into 6 cm culture plate at a density of 2.5×10^5 cells/plate for 24 h and then treated with PFPE at IC_{50} values. After treatment, cells were harvested every day for 4 days. Then, cell pellets were lysed using the RIPA buffer (containing 150 mM NaCl, 50 mM Tris, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate and 1 mM EDTA). Total protein samples (150 mg) were loaded on 12% of SDS-polyacrylamide gel electrophoresis and transferred onto a 0.45 mm nitrocellulose membrane (Bio-Rad, 162-0115). Membrane was blocked at room temperature for 1 h with 5% non-fat milk in 1X TBS-T and then washed with 1% non-fat milk in 1X TBS-T. Membrane was incubated with primary antibodies against topoisomerase II, Bcl-2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), p53 upregulated modulator of apoptosis (PUMA), p21, AKT8 virus oncogene cellular homolog (Akt), cyclooxygenase-2 (COX-2), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), signal transducer and activator of transcription 3 (STAT-3), cyclin D1 and p53 proteins. The membrane was then incubated with secondary horseradish peroxidase-conjugated antibodies. Bound antibodies were developed by a chemiluminescence detection kit using the SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific) and detected using a Fusion FX vilber lourmat, CCD camera (Fisher Biotechnology). GAPDH was used to normalize protein loading. Protein levels were expressed as a relative ratio to GPADH.

Statistical analysis

The median inhibition concentration (IC_{50}) data was acquired by SoftMax 1 Pro 5 program (MDS Analytical Technologies Inc., California, USA). Student's *t*-test was used to analyze intergroup differences. A $P < 0.05$ was considered to be statistically significant. All results were represented as the mean ± standard deviation (SD). The values were obtained from at least three independent experiments.

RESULTS

Total phenolic, tannin, and flavonoid contents

Phenolics, flavonoids, and tannins are one class of secondary plant metabolites which represented anticancer activity of plant. As present in Table 1, PFPE contained phenolic, tannin and flavonoid lower than methanol crude extract of *Curcuma longa* (MCLE). However, the cytotoxicity of PFPE against breast cancer MCF-7 cells (IC_{50} value at 7.45 ± 0.6 μg/ml) not significantly lower than MCLE (IC_{50} value at 5.74 ± 1.48 μg/ml). Therefore, we performed GC-MS in next experiment to identify the chemical compounds in PFPE.

Table 1: Total phenolic, tannin and flavonoid contents in piperine free *Piper nigrum* crude extract

Crude	Phenolics (mg GAE/g extract)[a]	Flavonoids (mg QE/mg extract)[b]	Tannins (mg CE/mg extract)[c]
PFPE	402.46±7.49	40.69±5.99	201.82±17.78
MCLE	2090.63±15.81	148.94±33.64	2373.75±92.77

[a]Mg of gallic acid equivalence by mg of extract; [b]Mg of quercetin equivalence by mg of extract; [c]Mg of catechin equivalence by mg of extract; *P. nigrum*: *Piper nigrum*; PFPE: Piperine free *P. nigrum* extract; *C. longa*: *Curcuma longa*; MCLE: Metanolic *C. longa* extract; GAE: Gallic acid equivalent; QE: Quercetin equivalent; CE: Catechin equivalent

Phytochemical screening

In this study, the phytochemical analysis using GC-MS was carried out. The chromatogram and predicted constituents are shown in Figure 1 and Table 2.

Results showed that PFPE contained five chemical groups including alkaloids, terpenes, amides, lignans, opioid and steroid with 17, 13, 7, 3, 1, and 1 compounds, respectively. The highest percentage of peak area of each group were pipercitine (21.66%, alkaloid), caryophyllene (13.28%, terpene), acrivastine (2.34%, amide), kusunokinin (1.28%, lignan), methyldihydromorphine (1.18%, opioid), and beta-stigmasterol (1.74%, steroid) which showed the anticancer activity.

Effect of piperine free *Piper nigrum* extract on the viability of cholangiocarcinoma, cholangiocyte and normal fibroblast cell lines

The cell viability of CCA and normal cell lines was measured using the MTT assay. All cell lines were incubated with extracts for 48 h. The IC_{50} values represented the mean ± SD of three different experiments. Among these cell lines, PFPE showed the highest cytotoxicity against KKU-M213 cells with IC_{50} value of 13.70 ± 1.14 μg/ml. Moreover, PFPE demonstrated cytotoxic effect stronger than dichloromethane *P. nigrum* crude extract (DPCE) (IC_{50} at 22.22 ± 0.26 μg/ml) and piperine (IC_{50} at 27.01 ± 0.36 μg/ml). The positive reference drug (doxorubicin) showed a very strong cytotoxic activity on normal and almost cancer cells. Surprisingly, doxorubicin showed same cytotoxic activity with PFPE against TFK-1 cells [Table 3].

Piperine free *Piper nigrum* extract induces deoxyribonucleic acid fragmentation on KKU-M213 and TFK-1 cells

A DNA fragmentation assay was used to determine whether the action of PFPE was associated with apoptosis or not. Apoptosis can be visualized as a ladder pattern of 180-200 base pairs due to DNA cleavage by the activation of a nuclear endonuclease enzyme. Since, PFPE demonstrated a strong cytotoxic effective on KKU-M213 and TFK-1 cells, both cell lines were used to determined DNA fragmentation. As shown in Figure 2, the DNA ladder pattern was observed at day 2 after exposure with 3 folds of IC_{50} concentration of PFPE.

Piperine free *Piper nigrum* extract inhibited proteins associated with inflammation that induces bile duct cancer

In this experiment, we determined proteins associated with inflammation that induced bile duct cancer including STAT-3, COX-2 and NF-kB using Western blot analysis. KKU-M213 cells were treated with 13.69 μg/ml of

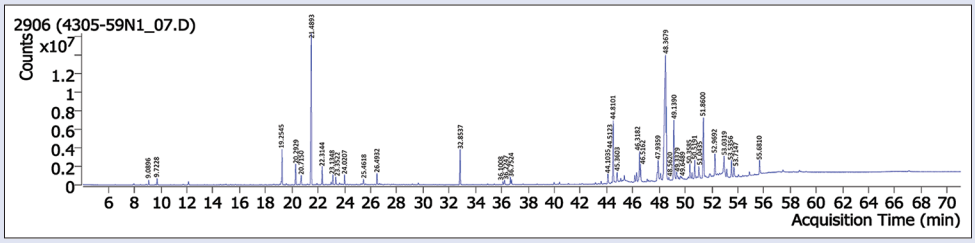


Figure 1: Gas chromatograph-mass spectrometer chromatogram of piperine free *Piper nigrum* extract

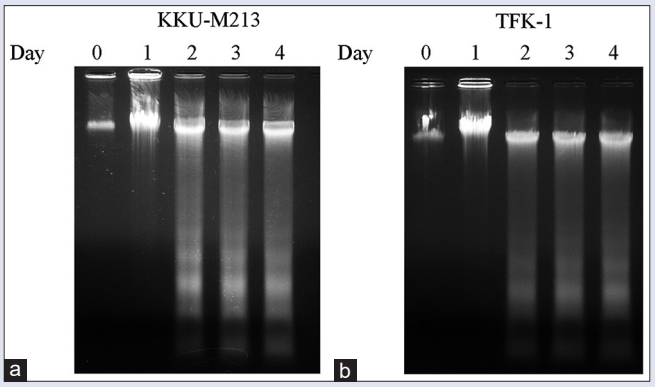


Figure 2: Analysis of Deoxyribonucleic acid fragmentation induced by piperine free *Piper nigrum* extract in KKU-M213 and TFK-1 cell lines. Cells were treated with piperine free *Piper nigrum* extract for 4 days and Deoxyribonucleic acid fragmentation was assessed by 1.5% agarose gel electrophoresis and ethidium bromide staining. KKU-M213 (a) and TFK-1 (b) cells were treated with 41.10 and 45.90 μg/ml of piperine free *Piper nigrum* extract, respectively. The data are representative of three independent experiments carried out under the same conditions

PFPE and incubated for 96 h. The results showed that the STAT-3, COX-2 and NF-kB protein levels were reduced in a time dependent manner and significantly decreased at 48-96 h [Figure 3a and c]. Furthermore, TFK-1 cells were treated with 15.29 μg/ml of PFPE and incubated for 96 h cells. The STAT-3 and COX-2 protein levels were significantly reduced at 72-96 h in a time-dependent manner. The NF-kB protein was decreased significantly at 24 and 72 h [Figure 3b and d].

Piperine free *Piper nigrum* extract inhibited proteins involved in the cell proliferation and growth

Proteins related to cell proliferation and growth of bile duct cancer cells, including topoisomerase II, Akt, c-Myc, cyclin D1, and p21 were examined after treatment with PFPE using IC_{50} concentration of each cells. The result showed that topoisomerase II was significantly decreased at 24 h and p21 was increased at 96 h in KKU-M213 cells [Figure 4a and c]. Meanwhile, PFPE treated TFK-1 cells showed a significant decreased in topoisomerase II at 72 h and p21 was increased at 24 h [Figure 4b and d]. Then, Akt protein was decreased at 48 and 72 h in KKU-M213 and TFK-1 cells, respectively. Moreover, c-Myc and cyclin D1, a protein that worked after those proteins, were found significantly decreased at 48-96 h in both cell lines [Figure 4].

Piperine free *Piper nigrum* extract inhibited proteins associated with apoptosis

In this study, proteins associated with apoptosis pathway including antiapoptosis (Bcl-2) and apoptosis (p53, bax, and PUMA) were evaluated. After giving PFPE at IC_{50} concentration for 48 h, death cells were observed and Bcl-2 was decreased in both cells, KKU-M213 and TFK-1 [Figure 5]. In addition, the levels of p53 and Bax proteins were significantly increased at 96 h and PUMA protein was increased from 24 to 48 h in KKU-M213 cells [Figure 5a and c]. Moreover, p53, Bax and PUMA were increased significantly at 24 h TFK-1 cells [Figure 5b and d].

DISCUSSION

The incidence of bile duct cancer or CCA has increased in Thailand and chemotherapy is not sufficient to treat the aggressive type of this cancer.[5] Therefore, medicinal plants could be an alternative treatment for bile duct cancer. There are many medicinal plants that cause cell cycle arrest and apoptosis in CCA such as *Tripterygium wilfordii*, *Atractylodes lancea* (Thunb) DC., *Zingiber officinale* Roscoe, *Phyllanthus emblica*, *Terminalia chebula* Retz., *Moringa oleifera*, and *Curcuma longa* Linn.[20,21] *Piper* species is one of medicinal plant that also shows anticancer effect, such as *Piper sarmentosum,*[22] *Piper longum,*[23] *Piper chaba*[24] and *P. nigrum.*[17] In previous study, we reported that PFPE showed anticancer activity against breast cancer in *in vitro* and *in vivo*.[15,16] Here, we further explored the biological activity of PFPE on bile duct cancer and found that PFPE exhibited anticancer activity against CCA cell lines, especially TFK-1 and KKU-M213, a moderate differentiation with p53 mutation and well differentiation CCA cells, respectively. Using GC-MS technique, many active phytochemicals were founded in PFPE including alkaloids, terpenes, amides, lignans, opioid and steroids. Pipercitine, guineensine, and pipersintenamide, (an alkaloid compounds) represented percentage of peak area at 21.66, 10.17, and 5.65%, respectively. Pipercitine shows toxicity against larvae of *Aedes aegypti,*[25] and guineensine has an anticancer property against the mouse lymphoma cell line L5178Y with IC_{50} values of 17.0 μM.[26] Pipersintenamide, isolated from *Piper sintenense* Hatus, shows anticancer activity against leukamia P-388 and promyelocytic leukemia HL-60 cell lines with IC_{50} values of 3.78 and 3.80 μg/ml.[27,28] Moreover, caryophyllene (13.28% in PFPE), a bicyclic natural sesquiterpene, exhibits antiproliferative effects against colorectal cancer cells (IC_{50} 19 μM) though clonogenicity, migration, invasion and spheroid formation.[29] A beta-stigmasterol (1.74% in PFPE), a steroid compound, demonstrates inhibitory effects with IC_{50} values of 11.14 and 18.28 μM against human myeloid leukemia K562 and prostate cancer PC3 cell lines, respectively.[30] In this recent study, we found a very potent compounds in the PFPE including piperlonguminine (4.77%), kusunokinin (1.28%), and cubebin (0.28%), which have been reported as anticancer agents.(-)-Kusunokinin and piperlonguminine, a natural lignan and alkaloid compounds, inhibited breast cancer cells (MCF-7 and MDA-MB-468) and colorectal cells (SW-620) through down-regulation of topoisomerase II and up-regulation of of p53, p21 protein levels.[31] (-)-Cubebin, a lignan compound, represents anticancer effect against myeloid leukemia, lung and nasopharyngeal cancer.[32] Interestingly, we found that PFPE showed stronger cytotoxicity against CCA cells than DPCE and piperine [Table 3]. However, piperine, the major alkaloid compound in *P. nigrum*, still remained in the PFPE

Table 2: Chemical constituents in piperine free *Piper nigrum* extract

Identified compounds	Formula	Nature of compound	Molecular massb (g/mol)	Retention time	Area (%)	Biological activity
3-Carene	$C_{10}H_{16}$	Terpenes	136.24	9.0896	0.28	Antioxidant, antihyperuricemic and anti-inflammatory[33]
D-Limonene	$C_{10}H_{16}$	Terpenes	136.24	9.7228	0.39	Enhanced the antitumor effect of docetaxel against prostate cancer cells[34]
Clohexane, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans) 2,4-diisopropenyl-1-methyl-1-vinylcy clohexane (or beta-Elemene)	$C_{15}H_{24}$	Terpenes	204.36	19.2545	2.20	Cytotoxic effect on K562 (leukemic) cells by the induction of apoptosis[35]
Copaene	$C_{15}H_{24}$	Terpenes	204.36	20.2929	1.26	Antimicrobial activity against an anaerobic microorganism *Prevotella nigrescens*[36]
2,4-diisopropenyl-1-methyl-1-vinylcyclohexane (beta-Elemene)	$C_{15}H_{24}$	Terpenes	204.36	20.7150	0.73	Cytotoxic effect on K562 (leukemic) cells by the induction of apoptosis[35]
Caryophyllene	$C_{15}H_{24}$	Terpenes	204.36	21.4893	13.28	Antioxidant, preventing lipidic oxidative damage and prevention of atherosclerosis;[37] antigenotoxic and santioxidant[38]
1,4,7,-Cycloundecatriene, 1,5,9,9-tetra methyl-, Z, Z, Z-	$C_{15}H_{24}$	Terpenes	204.36	22.3144	1.15	No activity reported
Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-,[4aR-(4a.alpha.,7.alpha.,8a.beta.)]- (or β-helmiscapene, beta-Selinene)	$C_{15}H_{24}$	Terpenes	204.35	23.1348	0.60	Antioxidant and cytotoxic activity against HT29 (colon cancer) cells;[39] cytotoxicity against KB (oral cancer), MCF-7 (breast cancer) and NCI-H187 (small cell lung cancer) cells[40]
2-Isopropenyl-4a, 8-dimthyl-1,2,3,4,4a, 5,6,8a-octahydronapthalene (or 7-Epi-alpha-Selinene)	$C_{15}H_{24}$	Terpenes	204.36	23.3522	0.54	Antimicrobial activity against *Bacillus subtilis* and *Candida albicans*[41]
delta-Cadinene	$C_{15}H_{24}$	Terpenes	204.37	24.0207	0.61	Induction of apoptosis and cell cycle arrest on OVACR-3 (ovarian cancer) cells[42]
Caryophyllene oxide	$C_{15}H_{24}O$	Terpenes	220.36	25.4618	0.42	Chemosensitizing agents for doxorubicin chemotherapy;[43] anticancer;[44] increased the efficacy of DOX in MDA-MB-231 (breast cancer) cells;[45] inhibit STAT3 signaling pathway[46]
Isospathulenol	$C_{15}H_{24}O$	Terpenes	220.37	26.4932	0.71	Cytotoxic effects against *Aspergillus niger*, *Artemia salina* and *Caenorhabditis elegans*[47]
2,4-Decadienamide, N-isobutyl-, (E, E)- (or Pellitorine)	$C_{14}H_{25}NO$	Amides	223.36	32.8537	2.28	Antibacterial, anticancer and anti-inflammatory[48]
Piperidine, 1-(1-oxo-3-phenyl-2-prope nyl)- (or piperidine, 1-Cinnamoylpiperidine)	$C_{14}H_{17}NO$	Alkaloids	215.29	36.1008	0.22	No activity reported
(2E,4E)-1-(Pyrrolidin-1-yl) deca-2,4-dien-1-one (or Iyeremide A, sarmentine)	$C_{14}H_{23}NO$	Alkaloids	221.34	36.2247	0.37	Cytotoxicity against CCRF-CEM (acute lymphoblastic leukemia), HL-60 (acute promyelocytic leukemia), PC-3 (prostate carcinoma), and HA22T (hepatoma) cells;[27] inhibit lipoxygenase (5-LOX) and cyclooxygenase-1 (COX-1) [49]
(2E,4E)-N-Isobutyldodeca-2,4-dienamide (or Dodecatetraenoic acid isobutylamide)	$C_{16}H_{29}NO$	Amides	251.41	36.7524	0.48	Inhibit allergic and inflammatory[50]
N-Benzylidene-4-fluoroaniline	$C_{13}H_{10}FN$	Alkaloids	199.23	44.1035	0.34	No activity reported
(E)-5-(Benzo[d][1,3]dioxol-5-yl)-1-(pi peridin-1-yl) pent-2-en-1one (or piperanine)	$C_{17}H_{21}NO_3$	Alkaloids	287.359	44.5123	0.88	Hepatoprotective effect[51]
Piperlonguminine	$C_{16}H_{19}NO_3$	Alkaloids	273.33	44.8101	4.77	Anticancer against breast cancer cells[31]
(E)-1-(Piperidin-1-yl) hexadec-2-en-1-one	$C_{21}H_{39}NO$	Alkaloids	321.54	45.3603	0.79	No activity reported
Piperine	$C_{17}H_{19}NO_3$	Alkaloids	285.34	46.3182	5.09	Anticancer against Hep-G2 (hepatocellular carcinoma)[52] and Hela (cervical cancer) cells[53]
(2E,4E,10E)-N-Isobutylhexadeca-2,4,10-trienamide	$C_{20}H_{35}NO$	Amides	305.50	46.5162	0.48	No activity reported
(2E,4E)-N-Isobutyloctadeca-2,4-dienamide (or Pipericine)	$C_{22}H_{41}NO$	Amides	335.58	46.6004	0.85	Hepatoprotective effect[54]
1-Benzyl-2-(1-ethoxycarbonyl-2-phenylethyl)-4,5-dihydroimidazole (Acrivastine)	$C_{22}H_{24}N_2O_2$	Amides	348.45	46.6023	2.34	No activity reported
(E)-7-(Benzo[d][1,3]dioxol-5-yl)-1-(pyrrolidin-1-yl) hept-6-en-1 one (or Methyldihydromorphine)	$C_{18}H_{23}NO_3$	Opioid		47.8646	1.18	No activity reported

Contd...

Table 2: Contd...

Identified compounds	Formula	Nature of compound	Molecular massb (g/mol)	Retention time	Area (%)	Biological activity
Pyrrolidine, 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]-, (E, E)- (or Pyrrolidine, Trichostachine, Piperyline)	$C_{16}H_{17}NO_3$	Alkaloids	271.32	47.9359	2.58	Antiproliferative effect, cycle arrest, induce apoptosis on MCF-7 cells and antitumor effect *in vivo*[55]
1H-Indene, 2-fluoro-2,3-dihydro-1-methoxy-, trans-(.+-.)-	$C_{10}H_{11}FO$	Amides		48.1182	0.66	No activity reported
(E)-1-(Piperidin-1-yl) octadec-2-en-1-one (or Pipercitine)	$C_{23}H_{43}NO$	Alkaloids	349.60	48.3679	21.66	Insecticidal activity[25]
(E)-7-(Benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl) hept-6-en-1one (or Piperolein A)	$C_{19}H_{25}NO_3$	Alkaloids	315.41	48.5620	0.24	No activity reported
(2E,6E)-7-(Benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl) hepta-2,6-dien-1-one (or Pipersintenamide)	$C_{19}H_{23}NO_3$	Alkaloids	313.39	49.1390	5.65	Cytotoxicity against CCRF-CEM (acute lymphoblastic leukemia), HL-60 (acute promyelocytic leukemia), PC-3 (prostate carcinoma), and HA22T (hepatoma) cells[27]
(2E,4E,14E)-N-Isobutylicosa-2,4,14-trienamide (or 2,4,14-Eicosatrienamide)	$C_{24}H_{43}NO$	Amides	361.61	49.3379	0.59	Cytoprotective activity on normal fibroblast L929 cells and hepatoprotective activity[54]
2-Furanol, 3,4-bis (1,3-benzodioxol-5-ylmethyl) tetrahydro- (or 2-Furanol, Cubebin)	$C_{20}H_{20}O_6$	Lignan	356.37	49.6489	0.28	Antiinflammatory;[56] anticancer[32]
Retrofractamide-A	$C_{20}H_{25}NO_3$	Alkaloids	327.42	50.3585	0.34	Larvicidal activity against *Culex pipiens* pallens, *Aedes aegypti* and *Aedes togoi*;[57] hepatoprotective effect[54]
2 (3H)-Furanone, 3,4-bis (1,3-benzodioxol-5-ylmethyl) dihydro-,(3R-trans)- (or (+)-Hinokinin, Cubebinolide)	$C_{20}H_{18}O_6$	Lignan	354.36	50.5191	1.13	Antiinflammatory;[58] antioxidant[59]
(E)-9-(Benzo[d][1,3]dioxol-5-yl)-1-(pyrrolidin-1-yl) non-8-en-1-one (or Pyrrolidine, Tricholeine)	$C_{20}H_{27}NO_3$	Alkaloids	329.44	50.7269	0.42	Antiproliferative activity against various cancer cells[60]
(3R,4R)-3-(Benzo[d][1,3]dioxol-5-yl methyl)-4-(3,4-dimethoxybenzyl) dihydrofuran-2 (3H) one (or Kusunokinin)	$C_{21}H_{22}O_6$	Lignan	370.40	51.0435	1.28	Anticancer;[31] insecticidal activity against *Virola sebifera* and fungicidal activity against *Leucoagaricus gongylophorus*[61]
(E)-9-(Benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl) non-8-en-1-one (or Piperolein B)	$C_{21}H_{29}NO_3$	Alkaloids	343.47	51.3920	1.03	Inhibitor of acyl CoA: Diacylglycerol acyltransferase for potential therapy for the treatment of obesity and type 2 diabetes[62]
(2E,4E,12E)-13-(Benzo[d][1,3]dioxol-5-yl)-N-isobutyltrideca-2,4,12-trienamide (or Guineensine)	$C_{24}H_{33}NO_3$	Alkaloids	383.53	51.8600	10.17	Antiinflammatory[63]
(2E,4E,6E)-7-(Benzo[d][1,3] dioxol-5-yl)-1-(piperidin-1-yl) hepta-2,4,6-trien-1-one (or Piperettine)	$C_{19}H_{21}NO_3$	Alkaloids	311.38	52.9692	0.31	Trypanocidal effects against epimastigotes and amastigotes of *Trypanosoma cruzi*[64]
(22E)-Stigmasta-5,22-dien-3-ol (or beta-Stigmasterol, Poriferasterol)	$C_{29}H_{48}O$	Steroid	412.70	53.0319	1.74	Induce DNA damage and cell death[65]
(2E,4E,8E)-9-(Benzo[d] [1,3] dioxol-5-yl)-1-(piperidin-1-yl) nona-2,4,8-trien-1-one (or Dehydropipernonaline)	$C_{21}H_{25}NO_3$	Alkaloids	339.47	53.5356	2.32	Coronary vasodilating activity[66]
gamma-Sitosterol (or clionasterol)	$C_{29}H_{50}O$	Terpenes	414.72	53.7147	0.48	Cytotoxicity against P388 (murine lymphocytic leukaemia) and HL60 (leukemia) cells[67]
(2E,4E,12E)-13-(Benzo[d][1,3]dioxol-5-yl)-Nisobutyltrideca-2,4,12-trienamide (or Guineensine)	$C_{24}H_{33}NO_3$	Alkaloids	383.53	55.6810		Antiinflammatory[63]

at 5.09% [Table 2]. Similarly, CP2 (PFPE) exhibited IC_{50} values of 7.45 ± 1.59 μg/ml in MCF-7 cell lines, which was better than DPCE (IC_{50} at 23.46 ± 1.10 μg/ml).[17] These results indicate that PFPE, less piperine, was a potential crude extract in anticancer.

O. viverrini excretory/secretory products and *O. viverrini* antigen induce the expression of TLR4, IL-6, IL-8, TLR2, NF-κB, iNOS and COX-2 causing damage to biliary epithelium.[68] In this current study, PFPE showed down regulation of NF-kB, STAT-3 and COX-2 proteins [Figure 2]. In cancer cells, NF-kB and STAT-3 are major transcription factors that regulate proliferation, inflammatory, angiogenesis, invasive and apoptosis resistance by induction of several proteins, such as cyclin D, cyclin E1, CDK2, CDK4, CDK6, c-myc, tumor necrosis factor alpha, interleukin-1 (IL-1), IL-6, IL-8, VEGF and MMP-9.[69] NF-kB and STAT-3 proteins are induced by IL-6 to stimulate COX-2 expression in the inflammation process and cell cycle,[70,71] which associate to CCA progression. Therefore, suppression of NF-kB, STAT-3 and COX-2 proteins cause cancer growth inhibition. Piperlongumine, an alkaloid from *P. longum* reduces NF-kB and c-Myc protein levels and inhibits binding of NF-kB with DNA at promoters in lymphoma cancer cells.[72] Moreover, piperlongumine also reduced the phosphorylation of JAK-1, JAK-2 and STAT-3 in gastric cancer cells.[73] Matrine, an alkaloid from *Sophora flavescens* Ait., significantly inhibits the viability by reduction the phosphorylation levels of JAK-2 and STAT3 proteins in CCA cells.[74] Curcumin, a natural extracted polyphenol from *C. longa*, also suppresses proliferation in human biliary cancer cells through inhibition of NF-kB, STAT-3 and JAK1 proteins.[75]

Table 3: Cytotoxicity of piperine free *Piper nigrum* extract against cholangiocarcinoma, cholangiocyte and normal mouse fibroblast cell lines

Cell lines	IC_{50} value±SD (μg/ml)			
	DPCE	Piperine	PFPE	Doxorubicin
CCA				
KKU-100	22.88±0.43	46.53±0.09	17.79±0.88	0.78±0.03
KKU-M213	22.22±0.26	27.01±0.36	13.70±1.14	1.75±0.02
KKU-M055	46.66±0.48	55.32±0.22	16.74±0.61	0.69±0.09
TFK-1	23.25±0.45	29.38±0.07	15.30±0.18	15.19±0.12
HuCC-T1	37.17±0.03	35.02±0.12	20.72±0.75	2.53±0.04
Normal cholangiocyte				
MMNK-1	33.25±0.28	60.68±0.72	19.65±0.26	0.62±0.05
Normal fibroblast				
L-929	No effect	No effect	45.53±0.50	0.20±0.01

P. nigrum: *Piper nigrum*; DPCE: Dichloromethane *P. nigrum* crude extract; PFPE: Piperine free *P. nigrum* extract; CCA: Cholangiocarcinoma; SD: Standard deviation

There are many evidences on genes and proteins which relate to bile duct cancer growth and progression, such as p53 mutation, inactivation of p21 and activation of Ras and MAPKs proteins.[76] Here, we found that PFPE could inhibit CCA cancer proliferation by decreasing of topoisomerase II, Akt, c-Myc, cyclin D1, and increasing of p21 protein levels [Figure 4]. Topoisomerase II is an enzyme involved in the DNA replication process that controls cell cycle with peaking at G2/M phase.[77] Therefore, down regulation of topoisomerase II by PFPE could induced DNA damage, interrupted cell growth and caused cell death on KKU-M213 and TFK-1 cells. Most of the clinically active agents, including etoposide (lignan) and doxorubicin (alkaloid) are topoisomerase inhibitors.[78] Previously andrographolide analogue 3A.1 from *Andrographis paniculata*, a diterpenoid lactone, induces cell cycle arrest by down-regulation of CDK6 and cyclin D1 in KKU-M213 cell lines.[79] Surprisingly, PFPE also exerted a significant reduction of Akt protein leading to decreasing of c-Myc and cyclin D1 and increasing of p21 levels [Figure 6]. Akt and cyclin D1 stimulate the cell cycle progression from G1/S phase to G2/M phase.[80] β-caryophyllene oxide, a terpene compound from *P. nigrum*, shows down-regulation of downstream of AKT pathway, including cyclin D1, COX-2 and VEGF and also up-regulation of p53 and p21 proteins in human prostate and breast cancer cells.[81]

In this study, we founded that the PFPE induced cell death by causing DNA fragmentation, increasing apoptotic proteins (p53, Bax and PUMA) and decreasing Bcl-2 protein levels [Figure 5]. p53, a tumor suppressor and transcription factor, is initially induced when DNA

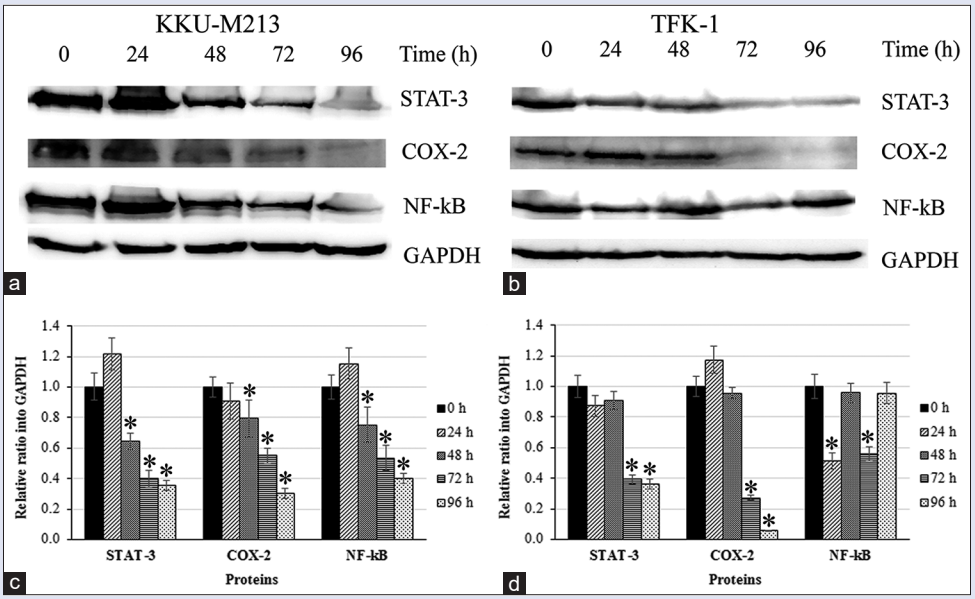


Figure 3: Expression of inflammation-related proteins in KKU-M213 (a and c) and TFK-1 (b and d) cells treated with piperine free *Piper nigrum* extract at 24, 48, 72 and 96 h. The levels of signal transducer and activator of transcription 3, cyclooxygenase-2 and Nuclear factor kappa-light-chain-enhancer of activated B cells and GAPDH proteins were measured using the Western blot analysis. Densitometric analysis normalized to GAPDH. Data were represented as mean ± standard deviation and three independent experiments were done. *$P < 0.05$ compared with control group (0 h)

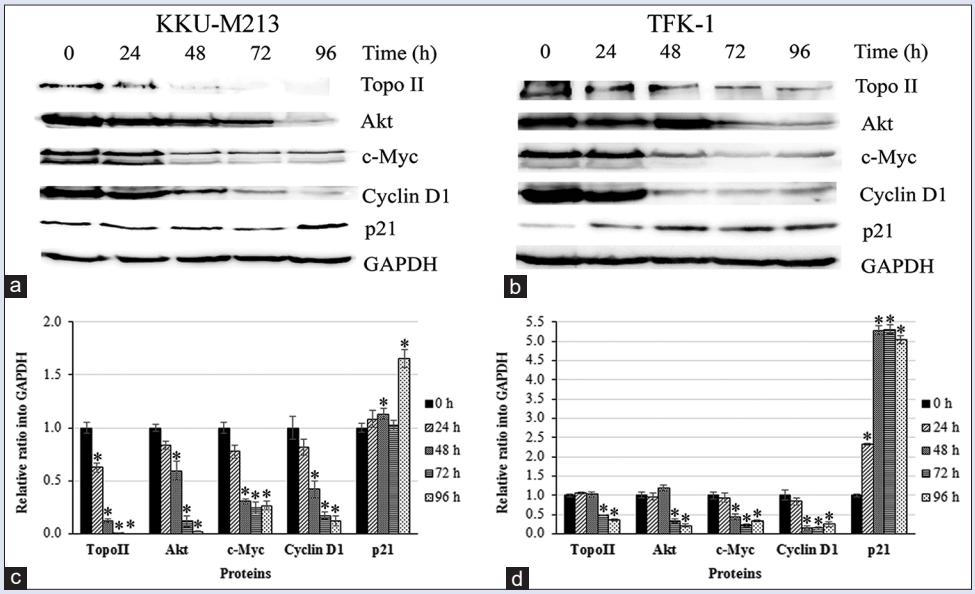


Figure 4: Effect of piperine free *Piper nigrum* extract on cell growth and cell cycle arrest. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of topoisomerase II, AKT8 virus oncogene cellular homolog, avian myelocytomatosis virus oncogene cellular homolog, cyclin D1 and p21 proteins were investigated using Western blot analysis. Fold change of each protein was measured by densitometry quantitation using ImageJ software and normalized with GAPDH. $P < 0.05$ of three independent experiments was considered to indicate a statistically significant differences compared to control group (0 h)

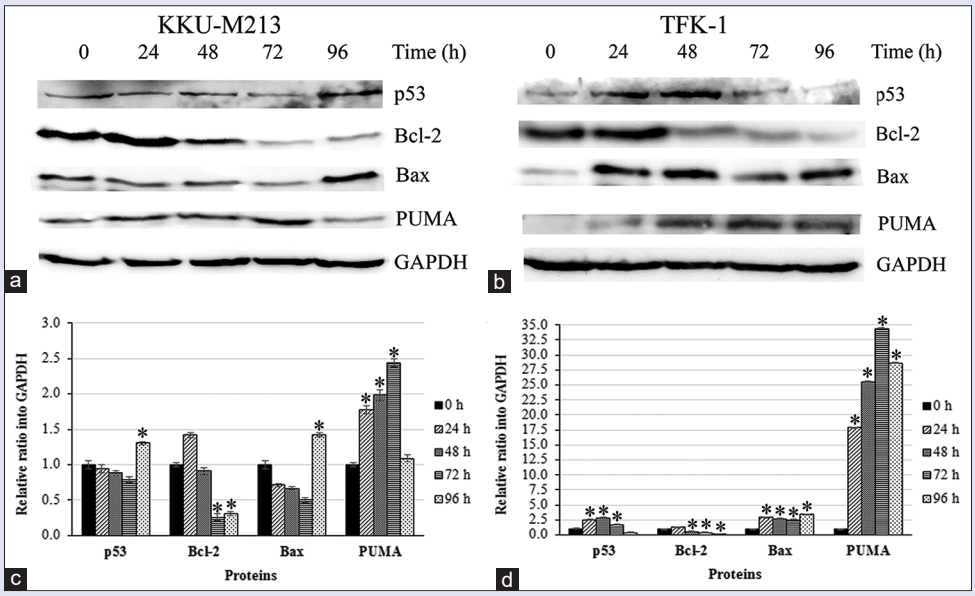


Figure 5: Effect of piperine free *Piper nigrum* extract on apoptosis. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of tumor protein p53, B-cell lymphoma 2, Bcl-2-associated X protein and PUMA proteins were investigated using Western blot analysis. Fold change of each protein was measured by densitometry quantitation using ImageJ software and normalized with GAPDH. $P < 0.05$ of three independent experiments was considered to indicate a statistically significant difference compared to control group (0 h)

damage and takes responsibility to activate several apoptotic genes, such as Bax, PUMA and NOXA.[82-84] Similarly, ethanolic extract of *P. nigrum* has antiproliferative effect on MCF-7 cells, antitumor effect *in vivo* and triggering apoptosis via p53 and Bax and decreasing of Bcl-2 proteins.[55] Curcumin effectively induces apoptosis in CCA (CCLP-1 and SG-231) cells by stimulation of Notch1, Hes-1 and survivin apoptotic proteins.[85] Andrographolide analog 3A.1 has cytotoxicity with IC_{50} of 8.0 μM on KKU-M213 cells at 24 h after treatment and induces apoptosis via induction of cleaved PARP-1, Bax, caspase-3, and p53.[79] Matrine stimulates apoptosis in CCA cells through induction of cytochrome c releasing from mitochondria and reduction of caspase-3 and-9 activity.[74] Taken together, PFPE can be a potential candidate for CCA treatment in future. However, study in CCA *in vivo* and clinical trial need to be carried out.

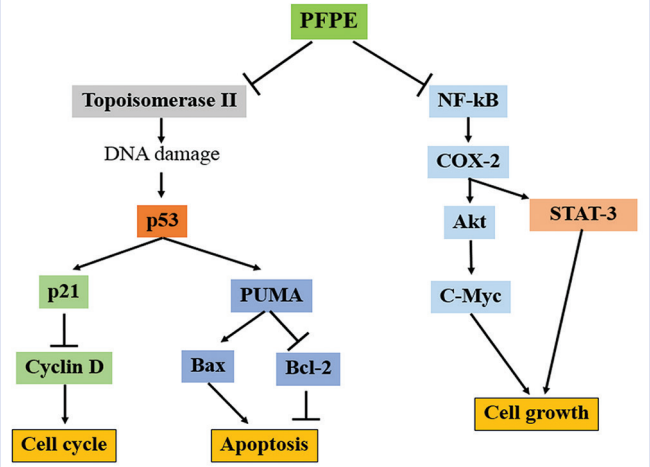


Figure 6: The anticancer mechanism of piperine free *Piper nigrum* extract in cholangiocarcinoma

CONCLUSION

PFPE showed strong cytotoxicity against KKU-M213 and TFK-1 cell lines with IC_{50} values of 13.70 ± 1.14 and 15.30 ± 0.18 µg/ml, respectively. PFPE suppressed inflammation through down-regulation of NF-kB, STAT-3 and COX-2. Moreover, PFPE inhibited CCA cells growth and proliferation by down-regulation of topoisomerase II, Akt, c-Myc and cyclin D and up-regulation of p21. Furthermore, PFPE triggered apoptosis through inhibition of Bcl-2 and induction of p53, Bax and PUMA levels as summarized in the Figure 5. In summary, PFPE can be served as a promising crude extract for CCA treatment.

Acknowledgement

The authors thank Prince of Songkla University for the equipment funding (the Excellent Research Laboratory (ExLab004)).

Financial support and sponsorship

This work was supported by the research fund (REC 60-279-04-2) Faculty of Medicine and the Postdoctoral Fellowship, Prince of Songkla University.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Blechacz B, Gores GJ. Cholangiocarcinoma: Advances in pathogenesis, diagnosis, and treatment. Hepatology 2008;48:308-21.
2. Ghouri YA, Mian I, Blechacz B. Cancer review: Cholangiocarcinoma. J Carcinog 2015;14:1.
3. Saha SK, Zhu AX, Fuchs CS, Brooks GA. Forty-year trends in cholangiocarcinoma incidence in the U.S.: Intrahepatic disease on the rise. Oncologist 2016;21:594-9.
4. Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. J Hepatol 2002;37:806-13.
5. Sripa B, Pairojkul C. Cholangiocarcinoma: Lessons from Thailand. Curr Opin Gastroenterol 2008;24:349-56.
6. Plentz RR, Malek NP. Clinical presentation, risk factors and staging systems of cholangiocarcinoma. Best Pract Res Clin Gastroenterol 2015;29:245-52.
7. Gatto M, Alvaro D. New insights on cholangiocarcinoma. World J Gastrointest Oncol 2010;2:136-45.
8. Squadroni M, Tondulli L, Gatta G, Mosconi S, Beretta G, Labianca R. Cholangiocarcinoma. Crit Rev Oncol Hematol 2017;116:11-31.
9. van der Gaag NA, Kloek JJ, de Bakker JK, Musters B, Geskus RB, Busch OR, *et al.* Survival analysis and prognostic nomogram for patients undergoing resection of extrahepatic cholangiocarcinoma. Ann Oncol 2012;23:2642-9.
10. Barreto JN, McCullough KB, Ice LL, Smith JA. Antineoplastic agents and the associated myelosuppressive effects: A review. J Pharm Pract 2014;27:440-6.
11. Ahmad N, Fazal H, Abbasi BH, Farooq S, Ali M, Khan MA. Biological role of *Piper nigrum* L. (Black pepper): A review. Asian Pac J Trop Biomed 2012;2:S1945-53.
12. Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jha A, *et al.* Phytochemistry of the genus *Piper*. Phytochemistry 1997;46:597-673.
13. Prashant A, Rangaswamy C, Yadav AK, Reddy V, Sowmya MN, Madhunapantula S. Anticancer activity of ethanolic extracts of & against colorectal carcinoma cell lines. Int J Appl Basic Med Res 2017;7:67-72.
14. Grinevicius VM, Andrade KS, Ourique F, Micke GA, Ferreira SR, Pedrosa RC. Antitumor activity of conventional and supercritical extracts from *Piper nigrum* L. cultivar Bragantina through cell cycle arrest and apoptosis induction. J Supercrit Fluids 2017;128:94-101.
15. Sriwiriyajan S, Tedasen A, Lailerd N, Boonyaphiphat P, Nitiruangjarat A, Deng Y, *et al.* Anticancer and cancer prevention effects of piperine-free *Piper nigrum* extract on n-nitrosomethylurea-induced mammary tumorigenesis in rats. Cancer Prev Res (Phila) 2016;9:74-82.
16. Deng Y, Sriwiriyajan S, Tedasen A, Hiransai P, Graidist P. Anti-cancer effects of *Piper nigrum* via inducing multiple molecular signaling *in vivo* and *in vitro*. J Ethnopharmacol 2016;188:87-95.
17. Sriwiriyajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. Cytotoxicity screening of plants of genus *Piper* in breast cancer cell lines. Trop J Pharm Res 2014;13:921-8.
18. Geran RI, Greenberg NH, McDonald MM, Schumacher AM, Abbot BJ. Protocol for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemoth Rep 1972;3:1-103.
19. Graidist P, Martla M, Sukpondma Y. Cytotoxic activity of *Piper cubeba* extract in breast cancer cell lines. Nutrients 2015;7:2707-18.
20. Plengsuriyakarn T, Thitapakorn V, Na-Bangchang K, Karbwang J. Thai medicinal plants: Potential sources of anticholangiocarcinoma drugs. Int J Pharm Pharmacol 2013;2:68-82.
21. Leelawat S, Leelawat K. Molecular mechanisms of cholangiocarcinoma cell inhibition by medicinal plants. Oncol Lett 2017;13:961-6.
22. Zainal Ariffin SH, Wan Omar WH, Zainal Ariffin Z, Safian MF, Senafi S, Megat Abdul Wahab R. Intrinsic anticarcinogenic effects of *Piper sarmentosum* ethanolic extract on a human hepatoma cell line. Cancer Cell Int 2009;9:6.
23. Ovadje P, Ma D, Tremblay P, Roma A, Steckle M, Guerrero JA, *et al.* Evaluation of the efficacy biochemical mechanism of cell death induction by *Piper longum* extract selectively in *in vitro* and *in vivo* models of human cancer cells. PLoS One 2014;9:e113250.
24. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in vitro*. BMC Complement Altern Med 2010;10:55.
25. Geris R, Ribeiro PR, Brandão MD, Da Silva HH, Silva IG. Bioactive natural products as potential candidates to control *Aedes aegypti*, the vector of dengue. In: Rahman A, editor. Studies in Natural Products Chemistry. Vol. 37. Oxford: Elsevier; 2012. p. 227-376.
26. Muharini R, Liu Z, Lin W, Proksch P. New amides from the fruits of *Piper retrofractum*. Tetrahedron Lett 2015;56:2521-5.
27. Chen JJ, Huang YC, Chen YC, Huang YT, Wang SW, Peng CY, *et al.* Cytotoxic amides from *Piper sintenense*. Planta Med 2002;68:980-5.
28. Chen JJ, Duh CY, Huang HY, Chen IS. Cytotoxic Constituents of *Piper sintenense*. Helv Chim Acta 2003;86:2058-64.
29. Dahham SS, Tabana YM, Iqbal MA, Ahamed MB, Ezzat MO, Majid AS, *et al.* The Anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of *Aquilaria crassna*. Molecules 2015;20:11808-29.
30. Shen T, Zhang L, Wang YY, Fan PH, Wang XN, Lin ZM, *et al.* Steroids from Commiphora mukul display antiproliferative effect against human prostate cancer PC3 cells via induction of apoptosis. Bioorg Med Chem Lett 2012;22:4801-6.
31. Sriwiriyajan S, Sukpondma Y, Srisawat T, Madla S, Graidist P. (-)-Kusunokinin and piperloguminine from *Piper nigrum*: An alternative option to treat breast cancer. Biomed Pharmacother 2017;92:732-43.
32. Rajalekshmi DS, Kabeer FA, Madhusoodhanan AR, Bahulayan AK, Prathapan R, Prakasan N, *et al.* Anticancer activity studies of cubebin isolated from *Piper cubeba* and its synthetic derivatives. Bioorg Med Chem Lett 2016;26:1767-71.
33. Kohoude MJ, Gbaguidi F, Agbani P, Ayedoun MA, Cazaux S, Bouajila J. Chemical composition

and biological activities of extracts and essential oil of Boswellia dalzielii leaves. Pharm Biol 2017;55:33-42.

34. Rabi T, Bishayee A. d -Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. J Carcinog 2009;8:9.
35. Zou L, Liu W, Yu L. Beta-elemene induces apoptosis of K562 leukemia cells. Zhonghua Zhong Liu Za Zhi 2001;23:196-8.
36. Martins Cde M, do Nascimento EA, de Morais SA, de Oliveira A, Chang R, Cunha LC, *et al.* Chemical constituents and evaluation of antimicrobial and cytotoxic activities of *Kielmeyera coriacea* Mart. & Zucc. essential oils. Evid Based Complement Alternat Med 2015;2015:1-9. doi.org/10.1155/2015/842047.
37. Baldissera MD, Souza CF, Grando TH, Stefani LM, Monteiro SG. β-caryophyllene reduces atherogenic index and coronary risk index in hypercholesterolemic rats: The involvement of cardiac oxidative damage. Chem Biol Interact 2017;270:9-14.
38. Alvarez-González I, Madrigal-Bujaidar E, Castro-García S. Antigenotoxic capacity of beta-caryophyllene in mouse, and evaluation of its antioxidant and GST induction activities. J Toxicol Sci 2014;39:849-59.
39. Ali NA, Wursterb M, Denkert A, Arnold N, Fadail I, Al-Didamony G, *et al.* Chemical composition, antimicrobial, antioxidant and cytotoxic activity of essential oils of *Plectranthus cylindraceus* and *Meriandra benghalensis* from Yemen. Nat Prod Commun 2012;7:1099-102.
40. Keawsa-ard S, Liawruangrath B, Liawruangrath S, Teerawutgulrag A, Pyne SG. Chemical constituents and antioxidant and biological activities of the essential oil from leaves of *Solanum spirale*. Nat Prod Commun 2012;7:955-8.
41. Ali NAA, Chhetri BK, Dosoky NS, Shari K, Al-Fahad AJ, Wessjohann L, *et al.* Antimicrobial, antioxidant, and cytotoxic activities of *Ocimum forskolei* and *Teucrium yemense* (*Lamiaceae*) essential oils. Medicines (Basel) 2017;4:7.
42. Hui LM, Zhao GD, Zhao JJ. δ-Cadinene inhibits the growth of ovarian cancer cells via caspase-dependent apoptosis and cell cycle arrest. Int J Clin Exp Pathol 2015;8:6046-56.
43. Dl Giacomo S, Dl Sotto A, Mazzanti G, Wink M. Chemosensitizing properties of β-Caryophyllene and β-caryophyllene oxide in combination with doxorubicin in human cancer cells. Anticancer Res 2017;37:1191-6.
44. Fidyt K, Fiedorowicz A, Strządała L, Szumny A. β-caryophyllene and β-caryophyllene oxide-natural compounds of anticancer and analgesic properties. Cancer Med 2016;5:3007-17.
45. Hanušová V, Caltová K, Svobodová H, Ambrož M, Skarka A, Murínová N, *et al.* The effects of β-caryophyllene oxide and trans-nerolidol on the efficacy of doxorubicin in breast cancer cells and breast tumor-bearing mice. Biomed Pharmacother 2017;95:828-36.
46. Kim C, Cho SK, Kapoor S, Kumar A, Vali S, Abbasi T, *et al.* β-Caryophyllene oxide inhibits constitutive and inducible STAT3 signaling pathway through induction of the SHP-1 protein tyrosine phosphatase. Mol Carcinog 2014;53:793-806.
47. Dosoky NS, Satyal P, Gautam TP, Setzer WN. Composition and biological activities of & *Murraya paniculata* (L.) Jack essential oil from Nepal. Medicines (Basel) 2016;3:7.
48. Ku SK, Lee IC, Kim JA, Bae JS. Anti-septic effects of pellitorine in HMGB1-induced inflammatory responses *in vitro* and *in vivo*. Inflammation 2014;37:338-48.
49. Stöhr JR, Xiao PG, Bauer R. Constituents of Chinese *Piper* species and their inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro*. J Ethnopharmacol 2001;75:133-9.
50. Gulledge TV, Collette NM, Mackey E, Johnstone SE, Moazami Y, Todd DA, *et al.* Mast cell degranulation and calcium influx are inhibited by an *Echinacea purpurea* extract and the alkylamide dodeca-2E,4E-dienoic acid isobutylamide. J Ethnopharmacol 2018;212:166-74.
51. Matsuda H, Ninomiya K, Morikawa T, Yasuda D, Yamaguchi I, Yoshikawa M. Protective effects of amide constituents from the fruit of *Piper chaba* on D-galactosamine/TNF-alpha-induced cell death in mouse hepatocytes. Bioorg Med Chem Lett 2008;18:2038-42.
52. Gunasekaran V, Elangovan K, Niranjali Devaraj S. Targeting hepatocellular carcinoma with piperine by radical-mediated mitochondrial pathway of apoptosis: An *in vitro* and *in vivo* study. Food Chem Toxicol 2017;105:106-18.
53. Han SZ, Liu HX, Yang LQ, Cui LD, Xu Y. Piperine (PP) enhanced mitomycin-C (MMC) therapy of human cervical cancer through suppressing Bcl-2 signaling pathway via inactivating STAT3/NF-κB. Biomed Pharmacother 2017;96:1403-10.
54. Matsuda H, Ninomiya K, Morikawa T, Yasuda D, Yamaguchi I, Yoshikawa M. Hepatoprotective amide constituents from the fruit of *Piper chaba*: Structural requirements, mode of action, and new amides. Bioorg Med Chem 2009;17:7313-23.
55. de Souza Grinevicius VM, Kviecinski MR, Santos Mota NS, Ourique F, Porfirio Will Castro LS, Andreguetti RR, *et al. Piper nigrum* ethanolic extract rich in piperamides causes ROS overproduction, oxidative damage in DNA leading to cell cycle arrest and apoptosis in cancer cells. J Ethnopharmacol 2016;189:139-47.
56. Lima TC, Lucarini R, Volpe AC, de Andrade CQJ, Souza AM, Pauletti PM, *et al. In vivo* and *in silico* anti-inflammatory mechanism of action of the semisynthetic (-)-cubebin derivatives (-)-hinokinin and (-)-O-benzylcubebin. Bioorg Med Chem Lett 2017;27:176-9.
57. Park IK, Lee SG, Shin SC, Park JD, Ahn YJ. Larvicidal activity of isobutylamides identified in *Piper nigrum* fruits against three mosquito species. J Agric Food Chem 2002;50:1866-70.
58. Godoy de Lima R, Barros MT, da Silva Laurentiz R. Medicinal attributes of Lignans extracted from & *Piper cubeba*: Current developments. ChemistryOpen 2018;7:180-91.
59. Medola JF, Cintra VP, Pesqueira E Silva EP, de Andrade Royo V, da Silva R, Saraiva J, *et al.* (-)-Hinokinin causes antigenotoxicity but not genotoxicity in peripheral blood of Wistar rats. Food Chem Toxicol 2007;45:638-42.
60. Wang YH, Goto M, Wang LT, Hsieh KY, Morris-Natschke SL, Tang GH, *et al.* Multidrug resistance-selective antiproliferative activity of *Piper* amide alkaloids and synthetic analogues. Bioorg Med Chem Lett 2014;24:4818-21.
61. Bicalho KU, Terezan AP, Martins DC, Freitas TG, Fernandes JB, Silva MF, *et al.* Evaluation of the toxicity of *Virola sebifera* crude extracts, fractions and isolated compounds on the nest of leaf-cutting ants. Psyche 2012;2012:785424.
62. Lee SW, Rho MC, Park HR, Choi JH, Kang JY, Lee JW, *et al.* Inhibition of diacylglycerol acyltransferase by alkamides isolated from the fruits of *Piper longum* and *Piper nigrum*. J Agric Food Chem 2006;54:9759-63.
63. Reynoso-Moreno I, Najar-Guerrero I, Escareño N, Flores-Soto ME, Gertsch J, Viveros-Paredes JM. An endocannabinoid uptake inhibitor from black pepper exerts pronounced anti-inflammatory effects in mice. J Agric Food Chem 2017;65:9435-42.
64. Ribeiro TS, Freire-de-Lima L, Previato JO, Mendonça-Previato L, Heise N, de Lima ME. Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of *Trypanosoma cruzi*. Bioorg Med Chem Lett 2004;14:3555-8.
65. Chaturvedula VS, Gao Z, Hecht SM, Jones SH, Kingston DG. A new acylated oleanane triterpenoid from *Couepia polyandra* that inhibits the lyase activity of DNA polymerase ß. J Nat Prod 2003;66:1463-65.
66. Shoji N, Umeyama A, Saito N, Takemoto T, Kajiwara A, Ohizumi Y. Dehydropipernonaline, an amide possessing coronary vasodilating activity, isolated from *Piper longum* L. J Pharm Sci 1986;75:1188-9.
67. Manoharan KP, Yang D, Hsu A, Huat BT. Evaluation of *Polygonum bistorta* for anticancer potential using selected cancer cell lines. Med Chem 2007;3:121-6.
68. Prueksapanich P, Piyachaturawat P, Aumpansub P, Ridtitid W, Chaiteerakij R, Rerknimitr R. Liver fluke-associated biliary tract cancer. Gut Liver 2018;12:236-45.
69. Fan Y, Mao R, Yang J. NF-κB and STAT3 signaling pathways collaboratively link inflammation to cancer. Protein Cell 2013;4:176-85.
70. Basu A, Das AS, Sharma M, Pathak MP, Chattopadhyay P, Biswas K, *et al.* STAT3 and NF-κB are common targets for kaempferol-mediated attenuation of COX-2 expression in IL-6-induced macrophages and carrageenan-induced mouse paw edema. Biochem Biophys Rep 2017;12:54-61.
71. Chen SC, Chang YL, Wang DL, Cheng JJ. Herbal remedy magnolol suppresses IL-6-induced STAT3 activation and gene expression in endothelial cells. Br J Pharmacol 2006;148:226-32.
72. Han SS, Son DJ, Yun H, Kamberos NL, Janz S. Piperlongumine inhibits proliferation and survival of Burkitt lymphoma *in vitro*. Leuk Res 2013;37:146-54.
73. Song B, Zhan H, Bian Q, Gu J. Piperlongumine inhibits gastric cancer cells via suppression of the JAK1,2/STAT3 signaling pathway. Mol Med Rep 2016;13:4475-80.
74. Yang N, Han F, Cui H, Huang J, Wang T, Zhou Y, *et al.* Matrine suppresses proliferation and induces apoptosis in human cholangiocarcinoma cells through suppression of JAK2/STAT3 signaling. Pharmacol Rep 2015;67:388-93.
75. Prakobwong S, Gupta SC, Kim JH, Sung B, Pinlaor P, Hiraku Y, *et al.* Curcumin suppresses proliferation and induces apoptosis in human biliary cancer cells through modulation of multiple cell signaling pathways. Carcinogenesis 2011;32:1372-80.
76. Fava G. Molecular mechanisms of cholangiocarcinoma. World J Gastrointest Pathophysiol 2010;1:12-22.
77. Larsen AK, Skladanowski A, Bojanowski K. The roles of DNA topoisomerase II during the cell cycle. Prog Cell Cycle Res 1996;2:229-39.
78. Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer 2009;9:338-50.
79. Nateewattana J, Dutta S, Reabroi S, Saeeng R, Kasemsook S, Chairoungdua A, *et al.* Induction of apoptosis in cholangiocarcinoma by an andrographolide analogue is mediated through topoisomerase II alpha inhibition. Eur J Pharmacol 2014;723:148-55.

80. Xu N, Lao Y, Zhang Y, Gillespie DA. Akt: A double-edged sword in cell proliferation and genome stability. J Oncol 2012;2012:1-15. doi:10.1155/2012/951724.
81. Park KR, Nam D, Yun HM, Lee SG, Jang HJ, Sethi G, *et al*. β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer Lett 2011;312:178-88.
82. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293-9.
83. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001;7:683-94.
84. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2001;7:673-82.
85. Koprowski S, Sokolowski K, Kunnimalaiyaan S, Gamblin TC, Kunnimalaiyaan M. Curcumin-mediated regulation of Notch1/hairy and enhancer of split-1/survivin: molecular targeting in cholangiocarcinoma. J Surg Res 2015;198:434-40.