Piper nigrum extract retards tumor growth by reducing tumor-promoted cytokines/chemokines and modulating immune cells in blood circulation

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Research Article

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Abstract

Piper nigrum, black pepper, has been widely used in traditional medicine to treat fevers and digestive system disease and is also applied to treat cancer in China. This study evaluated the breast cancer-preventive effect of a low piperine *Piper nigrum* extract (PFPE or LP-PE). Our findings showed that the incidence of tumors was 70% in the control group, 50% in the vehicle group, and 20% in the rats treated with PFPE at 50 and 100 mg/kg BW. Remarkably, no cancerous rats were found in the PFPE-treated at 150 mg/kg BW for approximately three months, with no significant changes in blood parameters, except for alkaline phosphatase (ALP). PFPE at 100 and 150 mg/kg BW suppressed cytokines/chemokines and increased ROS production compared to control and vehicle groups. PFPE stimulated IFN- γ promoted Th1 cells and inhibited Th2 and Treg compared to control and vehicle groups. In tumor-bearing rats, PFPE inhibited cancer progression by decreasing ER-α and NF-κB in the tumor tissue compared to control and vehicle groups. Our findings suggested PFPE has the potential to reduce tumor incidence and retardation of tumor growth by modulating Th1/Th2/Treg, ROS, cytokines/chemokines production and decreasing cancer-progression-related proteins in tumor rats.

1. Introduction

Breast cancer represents the main cause of death caused by cancer in women worldwide [1]. The development of breast cancer is regulated by genetic and epigenetic factors that regulate the interactions between tumor cells and the components of the tumor microenvironment (TME) [2]. Among these, immune cells play a dual potential for promoting tumor growth via immunoediting or suppressing cancer development by destroying transformed cells during carcinogenesis [2]. The immune system is categorized as innate and adaptive immune responses. Innate immune response recruits immune cells to the infection and inflammation sites through the production of chemokines, cytokines, and other mediators [3]. Adaptive immunity provides a specific immune response, including antigen-specific T cells. Over-expression or generating a protein by malignant cells that is foreign to the host immune system triggers T cell activation [4]. Therefore, enhancing the immune response to eradicate transformed cells has become a promising approach for cancer treatment and prevention [5].

Currently, cross-talk between natural compounds and the immune system demonstrates a promising approach to cancer prevention via the modulation of cells and components of innate and adaptive immunity [6]. Black pepper (*Piper nigrum* L.) is the most used household spice in the world, has been extensively used in folk medicine, is considered a traditional medicine [7], and has been found to possess hepatoprotective, anti-inflammatory, and anti-tumor [8]. Previous studies suggest that *P. nigrum* extract has potent immunomodulatory potential via the regulation of anti-inflammatory cytokines [9] and has potential immunomodulators of T helper (Th) 1/Th2 cytokines and enhances the cytotoxic of natural killer (NK) cells [10].

A crude extract of *P. nigrum*, namely low piperine fractional *Piper nigrum* extract (PFPE or LP-PE), had an anticancer effect on breast cancer both *in vitro* and *in vivo* [11, 12] through regulating various signaling

pathways. Moreover, PFPE enhanced the antitumor immunity via the regulation of Th1/Th2/Regulatory T (Treg) cells [13]. Combining PFPE with the chemotherapy drug (doxorubicin) improves blood parameters and reduces pro-tumorigenic cytokine induced by doxorubicin [14]. A recent study reported that PFPE reduces mammary tumor incidence and chemotherapy-induced toxicity [15].

Based on the efficacy of PFPE on the immune system that has been reported, this leads to the notion that enhancing immune responses by PFPE could be effective in breast cancer prevention or breast cancer retardation. Therefore, this study aimed to assess the protective effect of PFPE on NMU-induced mammary tumor rats and explore its immunological parameters, including cytokines, chemokines, T cells, and reactive oxygen species (ROS). Moreover, western blot was used to evaluate the level of protein-related cancer development in breast tumor rats.

2. Material and methods

2.1 Preparation of plant extract

Dried fruits of black peppercorn (*P. nigrum*) were collected from Nakhon Si Thammarat province of Thailand, identified by Assistant Professor Supreeya Yuenyongsawad, with the voucher specimen number SKP 146161401. PFPE extract was prepared using the previously described method [10]. Briefly, seventy grams of dry black peppercorn powder was macerated in 200 ml of dichloromethane (Sigma-Aldrich, US) for 3 h in a shaker incubator. The solution was then concentrated using a rotary evaporator (BUCHI, CH) at 45°C for 3 hours after being filtered using Whatman filter paper (No. 1) (Whatman Bioscience, UK). The dark brown oil residue of extracts was crystallized by adding 100 ml of cold diethyl ether. Then, yellow crystals (piperine) were filtered out using Whatman filter paper (No.3). The brown residue was then concentrated in a vacuum at 45°C for 3 h using a rotary evaporator. After that, the concentrated extract was stored in a desiccator until use. Each dose of PFPE was dissolved in a solvent (vehicle) prepared by mixing medium-chain triglyceride (MCT) with 2% vitamin E in distilled water.

2.2 Determination of PFPE contents by gas chromatography coupled with mass spectrometry (GC-MS)

The chemical composition of PFPE was analyzed using a Gas Chromatography-Agilent 7890B combination with an Agilent 5977A triple quadrupole mass spectrometer (Agilent Technologies Inc, CA) according to methods described previously [16]. The contents of PFPE were identified based on a correlation of the recorded fragmentation patterns of mass spectra provided in the GC-MS system software version Wiley10 and NIST14.

2.3 Tumor induction and PFPE feeding

Sixty female Sprague-Dawley rats (weighing 140–180 g), 45 days old, were obtained from Nomura Siam International Company Limited (Bangkok, Thailand). Animals were maintained at 22 ± 3˚C temperature, with an alternating 12-h light/dark cycle and 30–70% humid conditions in the Southern Laboratory

Animal Facility, Faculty of Science, Prince of Songkla University. The Ethics Committee approved all experimental animal protocols on the Animal experiment (Ref. 44/2019).

Rats were intraperitoneally injected with two doses of 250 mg/kg body weight of N-nitroso-N-methyl urea (NMU) (Sigma-Aldrich, US) at 50 and 80 days of age. After seven days of the second NMU injection, rats were fed 100 g/kg BW of estradiol (Sigma-Aldrich, US) for ten days, as previously described [13]. The rats were palpated three times per week to detect mammary tumor development. The rats were randomly divided into six groups (n = 10), including normal (healthy rats), control (received NMU injection without any treatment), vehicle (received NMU injection and a mixture of MCT oil and vitamin E in distilled water), PFPE50, PFPE100 and PFPE150 (received NMU injection and PFPE 50, 100 and 150 mg/kg BW, respectively). Apart from the normal group, rats were administered by gavage three times a week after 5 days of the 1st NMU injection and continued to be fed until the end of the experiment. The lateral tail vein was collected for cytokines/chemokines study during the experimental period at 15 and 61 days after PFPE treatment. At the end of the experiment, rats were sacrificed under anesthesia and blood was collected by cardiac puncture for hematological and biochemical parameters, cytokines/chemokines, ROS production and immune cell types. In addition, internal organs were weighed. Tumor tissues were collected for histology study and western blot analysis. The design of the experiment is summarized in Fig. S1.

2.4 Histopathological analysis

Through autopsy, the mammary tumor tissues from the NMU-induced rats were isolated. Afterward, breast tumor tissues were preserved in a 10% buffered formalin solution. The tissue was embedded in paraffin after fixation using a conventional automated system. The samples were spread out on glass slides after being sliced into 5-micron-thick slices. Before staining, the slides were deparaffinized in xylene, rehydrated in ascending graded ethanol series, and rinsed in distillate water. Then tumor tissues were stained with hematoxylin and eosin (H&E). Light microscopy was used to take serial paraffin sections of each tissue image at a magnification × 200 [10].

2.5 Assessment of Hematological and Biochemical Parameters

The blood was collected in a tube containing the EDTA anticoagulant to evaluate the complete blood count (CBC). The sample was performed on the automated hematology analyzer (ADVIA 120, Bayer Diagnostics, US) with optical laser light scattering for cell enumeration, flow cytometer and laser diffraction. Then, according to the manufacturer's instructions, blood was drawn into a clot activator tube for serum biochemistry analysis and run through an automated analyzer with an attached ion-selective electrode module (Cobas Mira, Roche Diagnostic Systems, CH).

2.6 Flow cytometry analysis of the subpopulation T-lymphocytes

The blood sample was derived from experimental rats by cardiac puncture. The blood sample was subjected to density gradient centrifugation to isolate PBMCs using Lymphoprep (Stemcell Technologies, CA). The obtained PBMCs (2×10^6 cells/mL) were then incubated with Leukocyte Activation Cocktail (BD GolgiPlug; BD Biosciences, CA) in completed RPMI-1640 medium for 4 h at 37 ˚C in a 5% CO_2 humidified incubator.

The cells were seeded in 96-well plates at a density of 2.5×10^5 cells per well. Then the cells were stained with FITC mouse anti-rat CD4 (0.5 mg/ml) and/or PE mouse anti-rat CD25 (0.2 mg/ml) antibodies (BD Biosciences, CA). Next, the cells were washed twice with permeabilization wash buffer (BD Biosciences, US) after being fixed with fixation buffer for 20 min at 4 ˚C. (BD Biosciences, US). Finally, the stained cells were incubated at four ˚C for 30 min in the dark with Alex 647 mouse anti-rat IFN-γ or PE mouse anti-rat IL-4 (0.2 mg/ml) antibodies (BD Biosciences, US). The stained cells were analyzed using the Beckman CytoFLEX S platform (Beckman Coulter, CA). The results were analyzed using Kaluza analysis flow cytometry software (Beckman Coulter, CA). All antibodies were stained with their isotype together as a baseline for comparison.

2.7 Measurement of cytokines profile

The blood sample was kept at 4 ˚C for not more than 24 h. Then blood was centrifuged at 1000×g at 4 ˚C for 20 min. Finally, the plasma was collected and stored at -80 ˚C until use. Rat cytokine antibody array was performed using a proteome profiler rat cytokine array kit, Panel A (Cat. ARY008, R&D System, CA). In one experiment, this assay kit's antibody-spotted membrane could detect 29 distinct cytokines/chemokines. The protocol was conducted following the manufacturer's guidelines. The membrane was detected by incubating with Chemi Reagent Mix for 1 min, and the signals were assessed upon exposure to peroxidase substrate using Alliance Q9 (Uvitec, Cambridge, UK). The intensity of the signal was scored using VITEC Alliance software (Uvitec, Cambridge, UK). Each spot of signal intensity was normalized to an internal control within the array and presented as the fold change responding to the normal group.

2.8 Thiobarbituric acid reactive substances (TBARS) assay

TBARS assay (Cat. STA-330, R&D Systems, CA) was used to evaluate the malondialdehyde (MDA) levels in each group of rats. MDA standard (0–64 mM) or sample volume of 50 microliters was mixed with 50 ml of SDS lysis solution and incubated at room temperature for 5 minutes. After adding the thiobarbituric acid (TBA) reagent, samples were incubated for 60 minutes at 95 ˚C. Next, the samples were centrifuged at 3000 g for 15 minutes after being held on ice for 10 minutes. The supernatant was transferred to a different tube, and n-butanol was added at an equal volume to avoid hemoglobin in the supernatant interfering. The mixed sample was vortex vigorously for 1–2 min and centrifuged at 10,000 g for 5 min. The butanol fraction was then transferred to a new tube for further measurement. The sample solution was measured at wavelength 532 nm by spectrophotometer (Thermo Fisher Scientific, Inc., US). The MDA concentration of the sample was calculated as previously described [11]. The MDA concentration of

samples was calculated using the equation obtained from the linear regression of the MDA standard curve, replacing the A532 nm values for each sample.

2.9 Western blot analysis

Proteins were extracted from tumor tissue using RIPA lysis buffer (Thermo Fisher Scientific, US) with a protease inhibitor (Sigma-Aldrich; Merck KGaA, DE). Each 20 µg of protein was separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% non-fat milk in 1X TTBS, membranes were incubated with specific primary antibodies, including IL-6R (Cat. 23457-1-AP, Proteintech Singapore Pte Ltd, SG), ER-α (Cat. Sc8002, Santa Cruz Biotechnology, Inc, US), NF-κB (Cat. 8242, Cell Signaling Technology, US) and STAT3 (Cat. 4904, Cell Signaling Technology, US) for 3 h. After washing with 1% non-fat milk in 1X TTBS, the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies for 1 h. After washing with 1X TTBS, the protein band was detected using a Super signal West Dura Extended Duration Substrate kit (Thermo Fisher Scientific, US) and detected using a Fusion FX CCD camera (Fisher Biotec, AU). GAPDH was used as the loading control. The protein level was expressed as a relative ratio to GAPDH.

2.10 Statistical analysis

The results were analyzed using SPSS, version 28.0.1.0 (IBM Corp., US). The results were expressed as mean ± SEM, except for serum MDA concentration was represented as mean ± SD. Significant differences between groups were assessed by one-way ANOVA followed by post-hoc least significant difference (LSD) test. $P < 0.05$ was considered statistically significant for cytokine array assay, flow cytometry, TBARS assay and Western blot analysis.

3. Results

3.1 Chemical composition of PFPE

The obtained extract appeared sticky, oily, and black. The yield of PFPE extract from the initial black peppercorns was approximately 2.2%. Only 14.99% of piperine was maintained in the extract as a result of the crystallization step in the extraction technique. GC-MS analysis of PFPE presented more than 40 compounds and constituents in 3 major groups, including alkaloids, terpene and lignan. The ion chromatograms of PFPE with immunomodulatory activity are shown in Fig. 1. Table S1 shows the 18 chemical substances found in the PFPE (each representing more than 1% of the total compounds) contributing to the anti-inflammatory, antioxidant, antibacterial anticancer, and immune activity.

3.2 PFPE reduces tumor incidence without causing negative side effects

This study aimed to determine the cancer-preventive effect of PFPE under carcinogenic exposure conditions. After 91 days of PFPE feeding, the result showed that the tumor incidence rate was 20% at 50 and 100 mg/kg BW doses of PFPE, and no tumor was found in PFPE at the dose of 150 mg/kg BW with no affect the organ to body weight ratio. Meanwhile, tumor incidence found in the control and vehicles groups was 70% and 50%, respectively (Table 1). H&E staining of the tumor tissues showed that the control and vehicle groups were invasive ductal carcinoma. The density of tumor cells differs from that of normal breast tissue, which has only two layers of cells surrounding the milk ducts. Whereas rats treated with 50 and 100 mg/kg BW of PFPE had ductal carcinoma in situ with less tumor cell density than the control group (Fig. S2).

Table 1

Tumor incidence, mean body weight, organ to body weight ratio, complete blood count and clinical chemistry parameters of the preventive study on the NMU-induced mammary tumorigenesis in rats.

Parameters	Normal	Control	Vehicle	PFPE 50 mg/kg	PFPE 100 mg/kg	PFPE 150 mgkg
% Tumor incidence	0	70	50	20	20	0
Body weght (g)	374.83 ± 20.62	364.20 ± 5.98	354.00 ± 10.03	377.00 ± 16.12	365.33 ± 9.83	352.17 ± 11.50
Oragan to body weight ratio						
Heart	0.28 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.32 ± 0.03	0.29 ± 0.01	0.30 ± 0.01
Liver	3.49 ± 0.10	3.62 ± 0.07	3.53 ± 0.07	3.70 ± 0.11	3.63 ± 0.11	3.62 ± 0.08
Lung	0.36 ± 0.01	0.39 ± 0.01	0.39 ± 0.01	0.37 ± 0.01	0.38 ± 0.01	0.38 ± 0.01
Spleen	0.16 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.18 ± 0.01	0.17 ± 0.00	0.17 ± 0.01
Kidney	0.62 ± 0.03	0.62 ± 0.02	0.61 ± 0.01	0.61 ± 0.02	0.61 ± 0.01	0.63 ± 0.01
Stomach	0.49 ± 0.04	0.57 ± 0.04	0.52 ± 0.03	0.48 ± 0.03	0.52 ± 0.03	0.52 ± 0.02
Complete blood count (CBC) parameters						
WBC ($x10^3$/µl)	2.58 ± 0.17	2.62 ± 0.32	1.98 ± 0.11[b]	2.25 ± 0.21	2.17 ± 0.18	2.40 ± 0.21
Neutrophil (%)	24.00 ± 2.28	40.17 ± 3.03[a]	31.67 ± 0.88[b]	35.00 ± 2.79[a]	33.33 ± 4.23[a]	29.67 ± 2.38[b]
Lymphocyte (%)	75.50 ± 2.36	57.83 ± 3.02[a]	66.17 ± 1.33[a,b]	63.33 ± 2.78[a]	65.17 ± 4.07[a]	68.83 ± 2.23[b]
Monocyte (%)	0.33 ± 0.33	0.83 ± 0.31	0.67 ± 0.21	1.17 ± 0.17[a]	0.83 ± 0.17	0.83 ± 0.31

Note: [a] presents $P < 0.05$, significantly different compared with the normal group. [b] presents $P < 0.05$, significantly different compared with the control group. [c] presents $P < 0.05$, significantly different compared with the vehicle group. WBC: White blood cells; RBC: Red blood cells; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

Parameters	Normal	Control	Vehicle	PFPE 50 mg/kg	PFPE 100 mg/kg	PFPE 150 mgkg
Eosinophil (%)	0.17 ± 0.17	1.17 ± 0.31[a]	1.50 ± 0.43[a]	0.50 ± 0.34[c]	0.67 ± 0.33	0.67 ± 0.21
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
RBC (x106/µL)	7.85 ± 0.24	7.58 ± 0.30	7.68 ± 0.13	7.54 ± 0.25	7.86 ± 0.12	7.73 ± 0.28
Hemoglobin g/dl)	14.90 ± 0.36	14.15 ± 0.35	14.65 ± 0.33	14.18 ± 0.23	14.70 ± 0.14	14.62 ± 0.45
Hematocrit (%)	45.67 ± 1.15	42.83 ± 1.42	45.17 ± 0.87	43.00 ± 1.15	45.00 ± 0.93	44.00 ± 1.59
MCV (fl)	58.33 ± 0.95	56.50 ± 0.76	58.50 ± 0.56	56.83 ± 0.87	57.00 ± 0.73	56.67 ± 0.61
MCH (pg)	19.00 ± 0.26	18.63 ± 0.44	19.08 ± 0.16	18.93 ± 0.45	18.73 ± 0.22	18.93 ± 0.20
MCHC (g/dl)	32.67 ± 0.16	32.73 ± 0.20	32.68 ± 0.22	33.27 ± 0.43	32.98 ± 0.33	33.38 ± 0.42
Platelet (x10^5/µL)	8.36 ± 0.28	8.41 ± 0.24	8.55 ± 0.24	8.94 ± 0.35	8.50 ± 0.37	8.62 ± 0.22
Clinical chemistry parameters						
BUN (mg/dl)	34.47 ± 0.84	30.28 ± 2.79	31.12 ± 1.31	32.55 ± 2.23	30.53 ± 1.49	29.28 ± 2.26
Creatinine (mg/dl)	0.43 ± 0.03	0.40 ± 0.06	0.51 ± 0.09	0.50 ± 0.07	0.49 ± 0.04	0.48 ± 0.04
Total protein (g/dl)	7.27 ± 0.11	7.05 ± 0.07	7.08 ± 0.20	7.62 ± 0.13	7.67 ± 0.07	7.63 ± 0.17
Albumin (g/dl)	4.15 ± 0.07	4.07 ± 0.05	4.08 ± 0.09	4.32 ± 0.07	4.32 ± 0.04	4.32 ± 0.07
Total bilirubin (mg/dl)	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.00	0.07 ± 0.01	0.07 ± 0.01

Note: [a] presents $P < 0.05$, significantly different compared with the normal group. [b] presents $P < 0.05$, significantly different compared with the control group. [c] presents $P < 0.05$, significantly different compared with the vehicle group. WBC: White blood cells; RBC: Red blood cells; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

Parameters	Normal	Control	Vehicle	PFPE 50 mg/kg	PFPE 100 mg/kg	PFPE 150 mgkg
AST (U/L)	105.50 ± 10.68	94.50 ± 13.16	94.67 ± 6.63	88.17 ± 8.31	96.83 ± 11.18	107.83 ± 11.32
ALT (U/L)	45.33 ± 6.21	34.67 ± 2.46[a]	39.83 ± 3.67	42.17 ± 3.48	43.50 ± 1.80	50.67 ± 2.35[b,c]
ALP (U/L)	98.83 ± 7.41	86.67 ± 9.54	75.67 ± 7.40	91.17 ± 8.01	83.50 ± 11.42	67.00 ± 5.09[a]
Cholesterol (mg/dL)	83.50 ± 4.17	111.50 ± 29.13	82.33 ± 3.89	80.00 ± 14.72	91.00 ± 8.05	84.83 ± 4.97
Triglycerides (mg/dL)	178.17 ± 14.14	179.67 ± 25.91	203.50 ± 10.18	233.17 ± 31.03	167.67 ± 32.84	154.67 ± 15.84

Note: [a] presents $P < 0.05$, significantly different compared with the normal group. [b] presents $P < 0.05$, significantly different compared with the control group. [c] presents $P < 0.05$, significantly different compared with the vehicle group. WBC: White blood cells; RBC: Red blood cells; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: Alkaline phosphatase.

In order to assess the impact of PFPE on systemic immune response, the number of certain types of circulating leukocytes was measured by flow cytometry. The results showed that PFPE at the dose of 150 mg/kg BW had the capacity to boost the population of lymphocytes and significantly suppressed the number of neutrophils compared to the control group. Interesting to notice that PFPE had no significant effect on the other complete blood count (CBC) parameters compared to the normal rats. Moreover, PFPE showed no significant differences in the clinical chemistry parameters compared to the normal group, except for ALP level in rats treated with 150 mg/kg BW of PFPE. Interestingly, all doses of PFPE had slightly lower cholesterol levels than the control group. Whereas only PFPE at the doses of 100 and 150 mg/kg BW had slightly decreased triglyceride levels compared to the control group. According to our findings, PFPE at doses of 50 and 100 mg/kg BW decreased the incidence of tumors and boosted the systemic immune response without any side effects on renal and liver function over the study period.

3.3 Effect of PFPE on cytokines/chemokines secretion

To support findings that PFPE can reduce tumor incidence via regulating the immune response, cytokine array analysis was used to assess the level of 29 associated cytokines/chemokines of breast cancer formation and progression. In this experiment, the level of cytokines and chemokines was determined using pool plasma samples from each group. After 15 days of PFPE feeding, the result showed that PFPE at doses of 50 and 150 mg/kg BW suppressed six cytokines and chemokines (sICAM-1, L-selectin, CX3CL1, CXCL7, VEGF, and TIMP1) involved in anti-inflammatory, immune response, and cancer development in comparison to control and vehicle groups. In contrast, 100 mg/kg BW of PFPE did not

decrease the cytokine and chemokine levels (Fig. 2A). However, 61 days of PFPE feeding, PFPE at 100 mg/kg BW had most efficiently decreased 12 cytokines and chemokines (Fig. 2B). At this point, there were found the number of cytokines and chemokines (5 chemokines and a cytokine) more than at the first point, including CCL5, CCL20, CXCL1, CXCL5, CXCL9, and IL-1β, which linked to the progression of cancer. At the end of the study (collected after 91 days of PFPE feeding), PFPE at the dose of 100 mg/kg BW also had the most effective suppressed 14 cytokines and chemokines. This point found three types of cytokines involved in T cell function, including IL-1α, IL-10, and IFN- γ. Interestingly, IFN- γ showed a high level in all doses of PFPE compared to the control and vehicle groups. Accordingly, these findings indicate that PFPE decreases tumor incidence by controlling the cytokines and chemokines involved in cancer development.

3.4 Effect of PFPE on quantification of $CD4^+$ helper T lymphocyte

To confirm the action of PFPE on the promotion of T lymphocytes in NMU-induced mammary tumor rats, the Th cell subtypes, including Th1 (tumor-suppressing), Th2 (tumor-promoting), and Treg (negative regulator immune response), were investigated using flow cytometry analysis. The results demonstrated that rats given PFPE at doses of 100 and 150 mg/kg BW had a significantly higher percentage of Th1 than the control and vehicle groups. Compared to the control and vehicle groups, the percentage of Th2 was significantly decreased in the PFPE100 and PFPE150 groups (Fig. 3). Moreover, the Treg in the PFPE150 group was significantly lower than in the control and vehicle groups. These findings indicate that PFPE modulated T lymphocytes in NMU-induced mammary tumor-induced rats by increasing the proportion of Th1 and decreasing Th2 and Tregs.

3.5 Effect of PFPE on pro-oxidant capacity

MDA concentration in plasma samples was used to assess the investigation into regulating ROS production of PFPE in NMU-induced mammary tumor rats. This study used a pool plasma sample of each group collected on sacrifice day to evaluate the MDA level. The MDA serves as an index for determining the extent of lipid peroxidation resulting from the breakdown of polyunsaturated fatty acids. Our results showed that rats were given PFPE at doses of 100 and 150 mg/kg BW significantly greater plasma MDA concentrations than the control and vehicle groups (Fig. 4). Accordingly, these findings indicate that PFPE induces cancer stress by generating ROS.

3.6 Effect of PFPE on levels of IL-6R, ER-α, STAT3 and NF-κB in NMU-induced breast tumor tissue

In breast cancer, ER-α and IL-6R activation can activate the NF-κB and STAT3 signaling pathways, leading to affecting all aspects of the tumorigenesis process by regulating proliferation, apoptosis, angiogenesis, and metastasis. In this study, the protein levels of ER-α, IL-6R, NF-κB, and STAT3 were assessed from the tumor tissue of one tumor rat in each group. The results demonstrated that PFPE at the dose of 50 and 100 mg/kg BW significantly increased the levels of IL-6R and STAT3 compared to the control and vehicle

groups. However, a PFPE-treated rat at a dose of 100 mg/kg BW had lower levels of IL-6R and STAT3 compared to rat treated with 50 mg/kg BW of PFPE (Fig. 5). In addition, PFPE treatment significantly reduced ER-α and NF-κB levels in comparison to the vehicle group. These findings suggest that PFPE reduced tumor growth by controlling protein-related cancer cell development.

Discussion

In this study, we performed approximately 3 months animal testing to evaluate the effect of PFPE on preventing breast cancer and modulating immune cells in blood circulation for future use in clinical trials. Our study used MCT oil instead of coconut oil that was used in previous study [15] for better solubility as MCT oil contains only medium chains while coconut oil contains medium and long-chain fat [17]. Vit E is an antioxidant that is used to prevent oil rancidity instead of honey. The main bioactive component found in *P. nigrum* is piperine. However, piperine was removed from the crude extracts by recrystallizing with cold diethyl ether in our study. According to the results of the GC-MS analysis (Table S1), bioactive compounds containing PFPE demonstrated anticancer [16, 18, 19], anti-inflammatory [20], and immune response-enhancing properties [21].

Our study found that PFPE-treated rats had a lower tumor incidence compared to the control and vehicle groups without significantly affecting the parameters related to renal and hepatic function compared to the normal group for three months. Noteworthy that PFPE at the dose of 150 mg/kg BW significantly decreased the ALP compared to the normal group. However, the low level of ALP was not a sign of liver damage [22]. For white blood cell parameters, increasing neutrophils reduces the number of lymphocytes and leads to impair immune cell function [23]. Our results showed that vehicle and PFPE150 groups significantly decreased neutrophil and increased lymphocyte counts compared to the control group.

Additionally, we verify that PFPE reduced tumor incidence via modulating the immune system response. Chemotaxis is a process wherein chemokines recruit leukocytes from circulation to the site of infection and inflammation [24]. The injection of NMU causes DNA damage and inflammation, which triggers the immunological response by releasing cytokines that draw in immune cells [25]. Our study found that after injecting NMU and receiving PFPE for 10 and 15 days, respectively. PFPE at a dose of 50 and 150 mg/kg BW reduced cytokines inflammation and immune response [26], Neutrophil trafficking inflammation [27], and tumor growth-promoting properties [28], including s-ICAM, L-selectin, CX3CL1, CXCL7, VEGF, and TIMP-1. These results suggest that PFPE effectively reduced chemokines/cytokines associated with inflammation and immune trafficking. After 31 days of PFPE feeding, PFPE inhibits six more cytokine/chemokine than point 1, which is related to supporting tumor cell proliferation and progression and the development of adaptive immunity [29]. These results might be cancer has occurred but were not detectable. However, cytokines/chemokines (CCL5, CCL20, CXCL9, and IL-1β) indicate the presence of tumors that can be detected in the plasma being examined at this point. At the endpoint of the experiment, cancer was detected in some rats. In pool plasma samples, the results found that PFPE decreased the levels of two cytokines involved in T cell function, including IL-10 and IL-1α. The IL-10 increases tumor growth by inhibiting IFN-γ secretion by Th1 and cytokine production by activated

macrophages and dendritic cells [30]. Moreover, IL-1α has a pro-tumorigenic response by the accumulation of tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) within the tumor microenvironment, which in turn promotes the differentiation of Tregs [31]. Interestingly, PFPE increased IFN-γ, which is involved in the anti-tumor response via activates NK cells, upregulates cell surface MHC class II on APCs, and increases the phagocytic ability of macrophage [32]. These results indicate that PFPE reduced the levels of cytokines and chemokines at all three points of the experiment. However, the tumor is present at the end of the experiment, PFPE may only prevent tumor growth for a shorter period than 91 days.

Adaptive immune responses are highly specific to the pathogen that induced them and provide long-lasting protection. We have performed the effect of PFPE on the T cell population in the blood sample at the endpoint of the experiment. Th1/Th2 balance is involved in the cancer immune response [33]. Th1 cells produce IFN-γ, IL-2, TNF-α, and IL-12, which have anticancer effects and stimulate macrophages to cause them to express MHC-I and II molecules, which improve their antigen presentation ability [34]. On the other hand, Th2 cells mediate the humoral immune and produce IL-4 and IL-10 to promote cancer progression. Moreover, IL-4 secreted by Th2 cells inhibits the secretion of IFN-γ [35]. Thus, the shifting from Th1 to Th2 response promotes breast cancer progression [36]. This study found that PFPE significantly increased the number of Th1 while decreasing Th2 cells compared to the control and vehicle groups. Tregs play an important role in controlling the balance between pro- and anti-inflammatory immune responses [37]. In the present study, PFPE at the dose of 150 mg/kg BW significantly decreased the frequencies of Tregs compared to the vehicle group, indicating that PFPE can halt the immunosuppression caused by Tregs in tumor-bearing rats. These results indicate that PFPE can modulate the immune response in NMU-induced mammary tumor rats via induced Th1 and decreased Th2 and Treg cells. However, the mechanism by which PFPE modulates the immune cells remains unclear. Therefore, further studies should investigate the mechanism underlying PFPE on T cell modulation.

ROS acts as a double-edged sword for tumors. ROS maintenance to a certain level is necessary and required for cellular proliferation and survival [38]. On the other hand, the rise in ROS can promote cancer cell death, inhibit cancer cell angiogenesis, and prevents immune escape in TME [39]. In this study, PFPE increased MDA levels compared to control and vehicle groups. The results had consistent with our previous studies, which showed that PFPE treated group induced apoptosis by inducing cells to produce ROS [11]. Moreover, the results of this study were consistent with the increase in IFN-γ induced by PFPE. The IFN-γ stimulates the production of ROS from macrophages, which leads to the destruction of cancer cells [40].

We further investigated the protein-related cancer development in tumor tissue of detectable tumor rats. ERα is critical to the development of ER + breast cancer [41]. Overexpression of ERα frequently sensitizes tumors to endocrine therapy [41]. ER-α activation stimulates downstream MAPK/ERK, PI3K/AKT, and p38/MAPK signaling pathways [42]. In addition, it has been reported that ER-positive human breast cancer cells express the IL-6 receptor and that their proliferation is inhibited by the addition of IL-6 [43].

The activation of IL-6R leads to promoting cancer cell proliferation, tumor invasion, and immune evasion via its downstream signaling, including STAT3 and NF-kB [44]. Our study showed that PFPE treatment significantly elevated the level of IL-6R and STAT3 compared to the control and vehicle groups. However, PFPE 100 mg/kg BW significantly decreased both proteins compared to PFPE 50 mg/kg BW. According to these findings, a low dose of PFPE might not be enough to block the protein. Also, a decrease in STAT3 was observed in cases where the cancer was more aggressive [45]. The results were consistent with our study in which the control and vehicle groups were invasive ductal carcinoma, while PFPE-treated were ductal carcinoma in situ. Moreover, PFPE treatment significantly decreased ER-α and NF-κB levels compared to the control and vehicle groups. These findings indicate that PFPE inhibits tumor growth via inhibiting protein-related cancer development.

Conclusion

Our finding reported that PFPE had a breast cancer preventive effect through the regulation of cytokines/chemokines, Th1/Th2/Treg cells, and ROS (Fig. 6). In case of the absence of cancer prevention, PFPE still suppressed cancer-associated protein development, resulting in tumor tissue in PFPE-treated less aggressively than the tumor in the control and vehicle groups. However, PFPE at a dose of 150 mg/kg BW should be carefully used for a long time due to its adverse effect on liver function.

Declarations

Author contributions NM: formal analysis, investigation, methodology, writing-original draft. ST, SD, JS, AN, TR, TT and CCN: they participated in the experimental study. PH: writing-review and editing. PG: conceptualization, supervision, validation, methodology, writing-review and editing.

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Data availability statement The datasets generated during and/or analysed during the current study are available from the corresponding author on a reasonable request.

Conflict of interest The authors declare no conflicts of interest.

Ethics approval This project was approved by the Ethical Committee of Prince of Songkla University, reference number 44/2019, date 10 June 2019.

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Figures

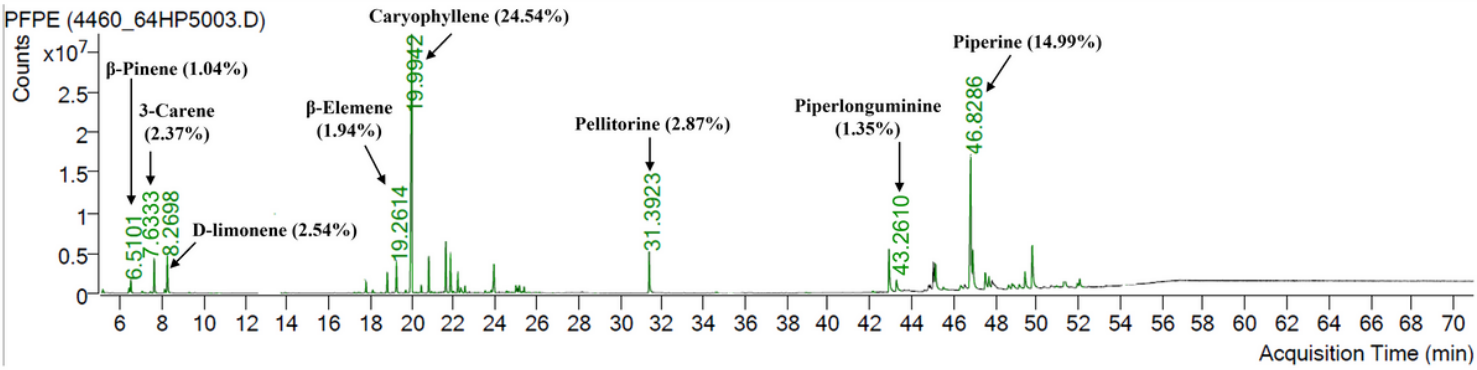

Figure 1

Chromatograms of the major compound in PFPE using GC-MS. The figure shows the retention times and percentages of compounds with immunologic activity

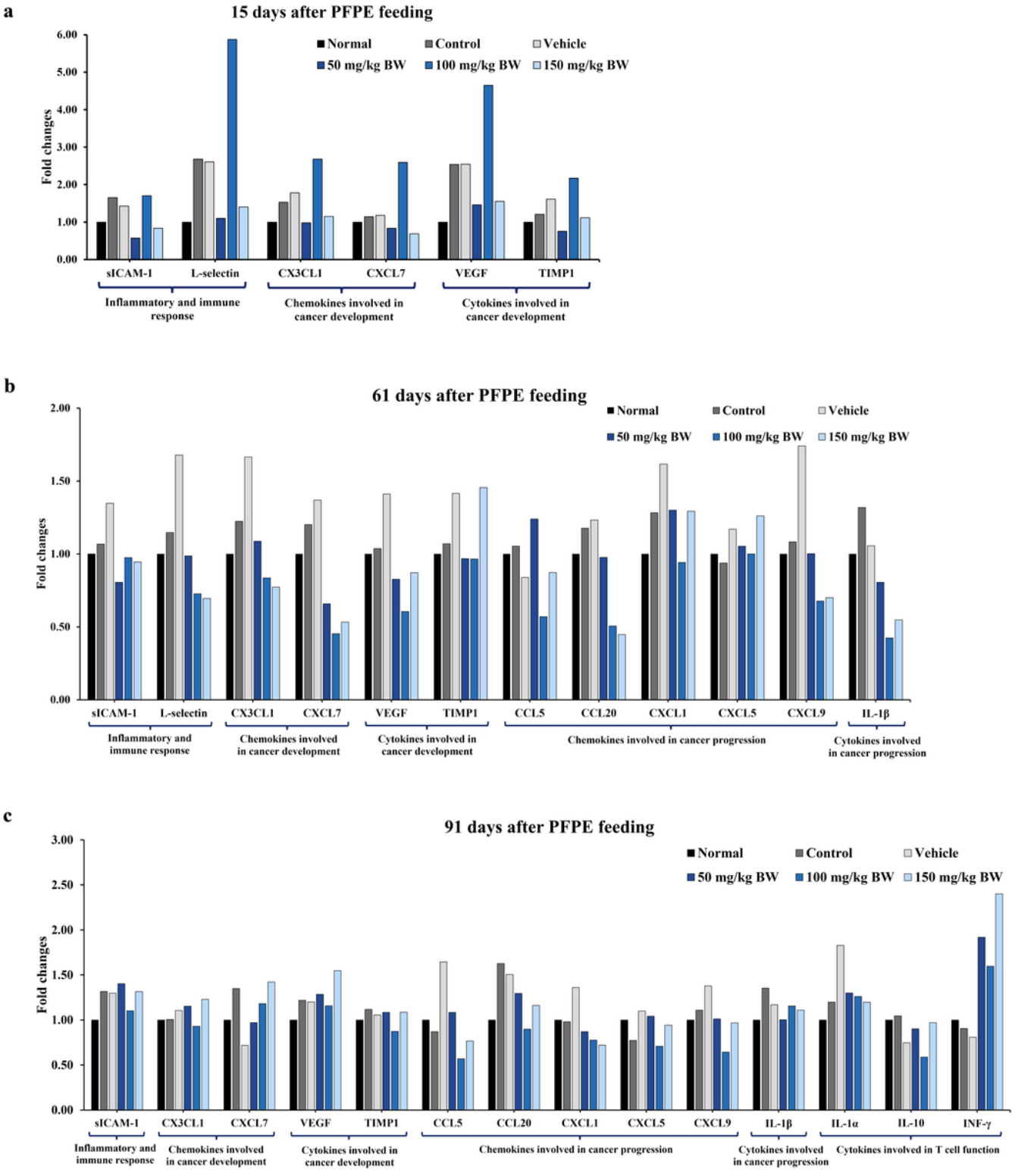

Figure 2

Effects of PFPE extract on blood cytokine/chemokine levels in rats with NMU-induced mammary tumors. Using the cytokine array assay, pool plasma samples from each group were utilized to assess the levels of cytokines and chemokines. **a** Results from 1st point of the experiment; after PFPE feeding 15 days, **b** Results from 2nd point of the experiment; after PFPE feeding 61 days and **c** The results obtained from the 3rd point of the experiment; after PFPE feeding 91 days

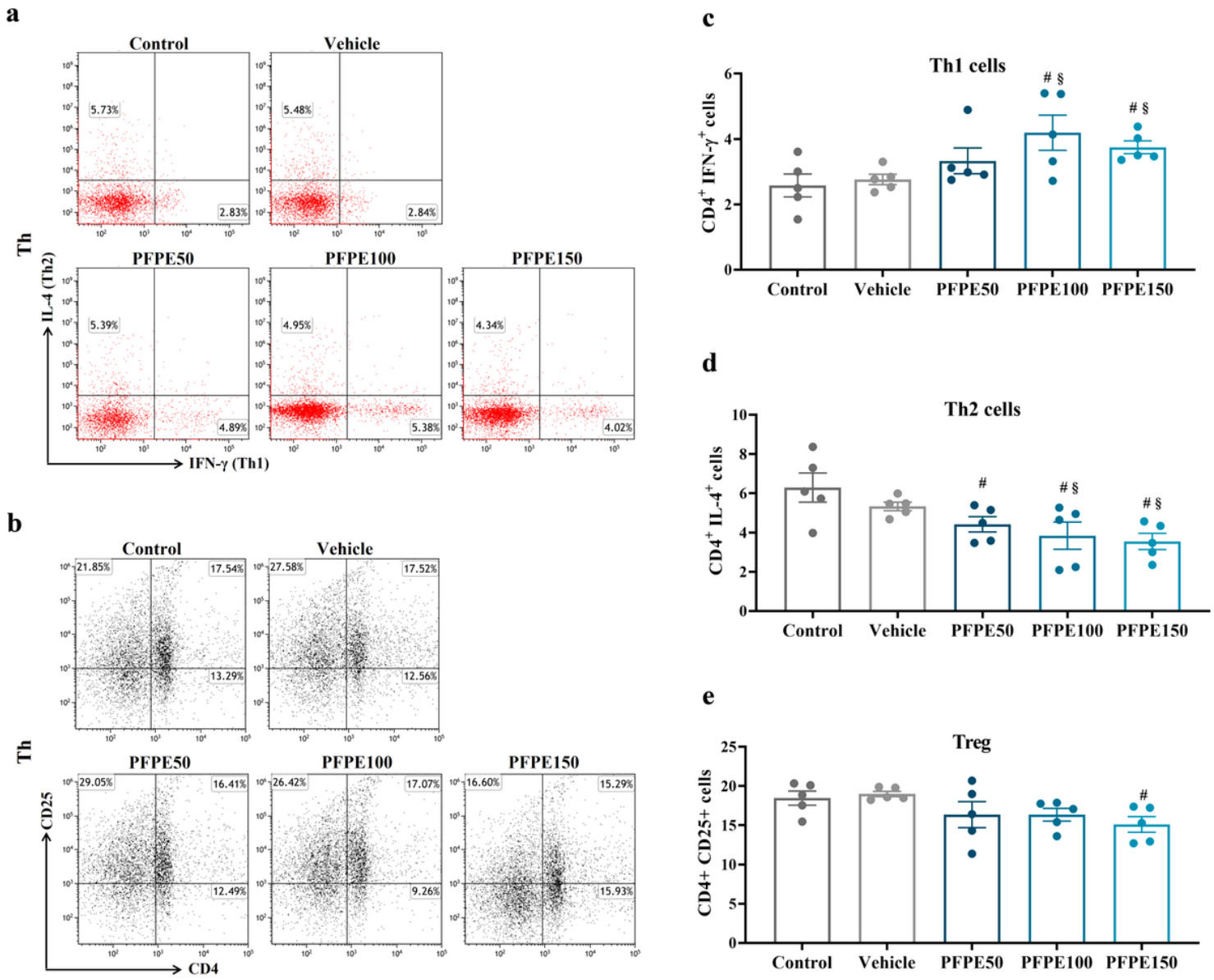

Figure 3

Flow cytometry analysis of circulating type 1 helper T cells (Th1), Th2 and Treg in NMU-induced mammary tumor rats treated with PFPE at the dose of 50, 100 and 150 mg/kg BW. **a** The representative dot plot of Th1 (CD4+IFN-γ+) and Th2 (CD4+IL-4+) proportions of each group of rats. **b** The representative dot plot of Treg (CD4+CD25+) proportions of each group of rats. **c,d,e** The percentage of Th1, Th2 and Treg, respectively. Data were expressed as mean ± SEM (n = 6). # $P < 0.05$, compared with the control group. § $P < 0.05$, compared with the vehicle group, one-way ANOVA followed by post-hoc LSD test

Figure 4

Effect of PFPE on oxidative stress marker in plasma MDA concentration of NMU-induced breast cancer rats. The pool plasma sample of each group was used to evaluate the level of MDA using TBARS assay. Data were expressed as mean ± SD of independent experiments. # P < 0.05, compared with the control group. § P < 0.05, compared with the vehicle group, one-way ANOVA followed by post-hoc LSD test

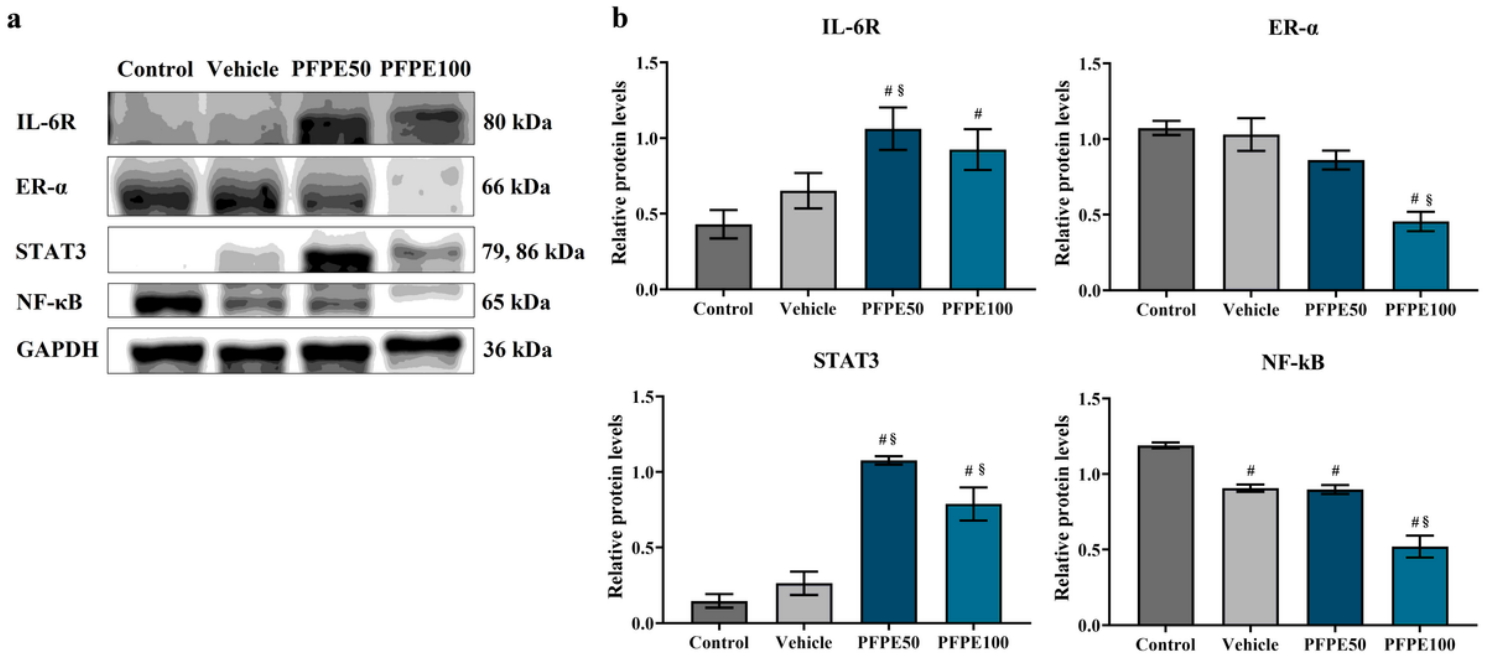

Figure 5

Expression of proliferation and inflammation-related protein in breast cancer tissue treated with PFPE at the end of experiment. **a** A tumor tissue of each group was used to measure the levels of IL-6R, ER-α, STAT3, NF-κB and GAPDH protein using the western blot analysis. **b** The semi-quantitative protein level was calculated by normalization with GAPDH band intensity. Data were expressed as mean ± SEM (n=6). # P < 0.05, compared with the control group. § P < 0.05, compared with the vehicle group, one-way ANOVA followed by post-hoc LSD test

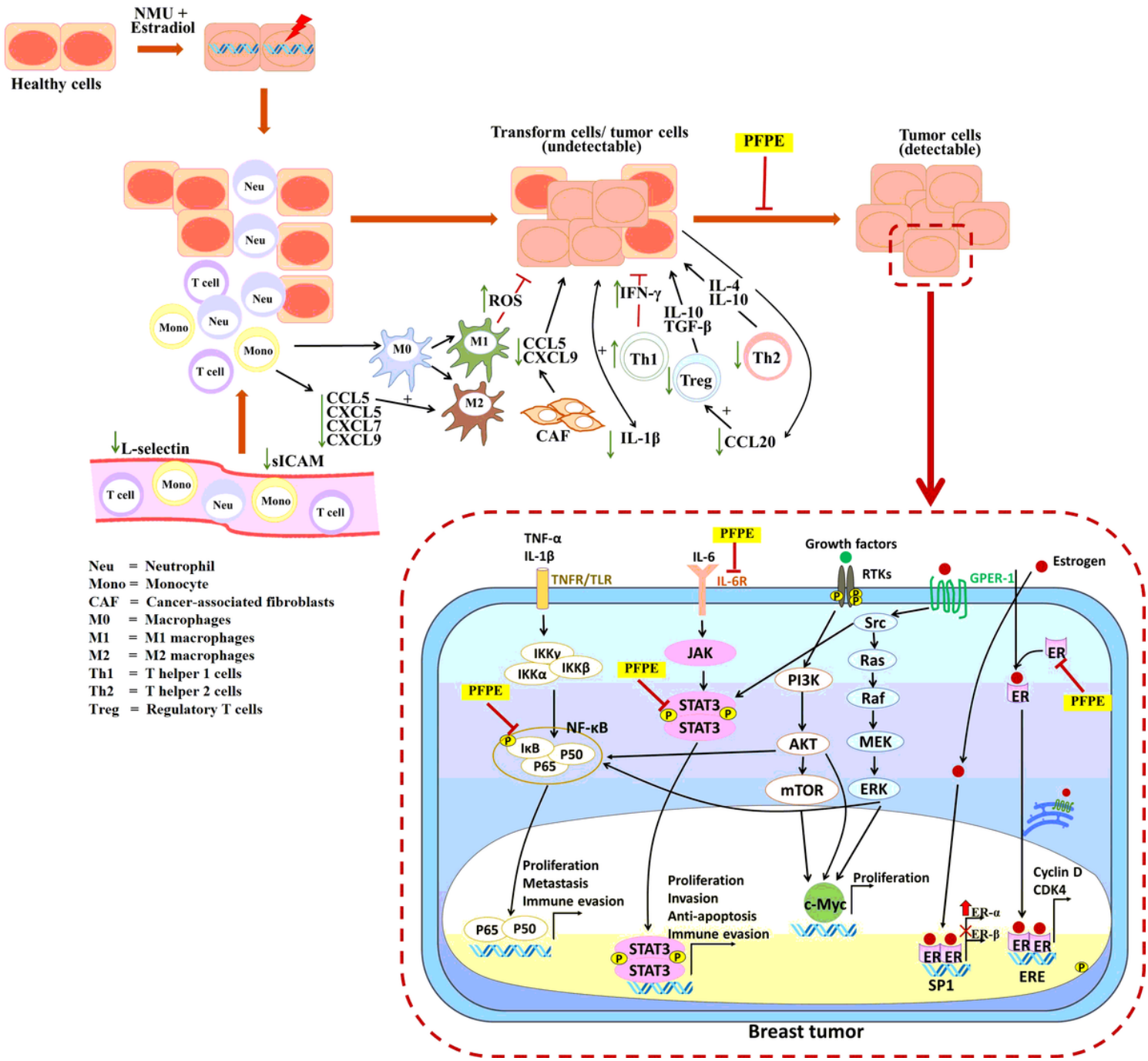

Figure 6

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