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Research article

Application of film-forming solution as a transdermal delivery system of piperine-rich herbal mixture extract for anti-inflammation

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ABSTRACT

Piperine-rich herbal mixture (PHM) used in this study is a traditional Thai medicine that contains 21 oriental herbs. It is called "Sahastara remedy" and is officially included in the Thai National List of Essential Medicine since A.D. 2011. PHM has been used orally to relieve muscle and bone pains. It contains *Piper nigrum* fruits as a major constituent and also *Piper retrofractum* fruits, PHM thus has anti-inflammatory activities that mostly come from the bioactivities of piperine consisting of these pepper fruits. Unfortunately, PHM usually causes gastrointestinal side effects. Consequently, a topical product containing an alcoholic extract of PHM (PHM-E), i.e., film-forming solution (FFS) was developed to overcome this drawback. The aims of this study were to investigate the anti-inflammatory activity of PHM-E, to evaluate physicochemical properties and the anti-inflammatory activity of FFS containing PHM-E (PHM-E FFS). Anti-inflammatory activities of PHM-E were investigated in the RAW 264.7 cells. Physicochemical properties, *in vitro* toxicities and anti-inflammatory activities of PHM-E FFS including its dry film (PHM-E film) were determined. PHM-E showed anti-inflammatory activities with dose dependent manners via inhibition of nitric oxide and prostaglandin E_2 production by the RAW 264.7 cells and promotion of the cell phenotype polarization from M1 to M2. PHM-E FFS had low viscosity and exhibited the Newtonian behavior. It provided elastic PHM-E film with low tensile strength. The release profile of piperine from PHM-E film followed a zero-kinetic model. PHM-E FFS demonstrated compatibility with the skin cells, minimal ocular irritant when accidentally splashing into the eye and moderate-to-high potency for inhibition of inflammatory symptoms in the rats. PHM-E FFS thus had potential for use in the further clinical study to investigate its efficacy and safety in patients.

1. Introduction

Inflammation is a pathophysiological response of living tissues to injuries, chemicals, irradiations, microbial infections and immune reactions. Its important signs are swelling, erythema, increased heat and pain at inflamed organs [1, 2, 3]. Recently, Global Burden of Disease 2016 (GBD 2016): Disease and Injury Incidence and Prevalence Collaborators [4] reported that during A.D. 2006–2016, the percentage changes of Years Lived with Disability (YLDs) of patients suffering from osteoarthritis (OA), low back pain and neck pain in 195 countries and territories grouped within 21 regions were increases of 31.5%, 18.0% and 21.9%, respectively. OA, in particular, causes knee pain and lower extremity disability in older adults and thus, lower quality of life [5].

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of acidic substances used for relieving inflammatory symptoms, pain and fever. They inhibit functions of cyclooxygenase enzymes (COXs) leading to blocking the synthesis process of prostaglandins. NSAIDs are used worldwide by more than 30 million people [6]. Around 59% of OA

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patients were treated with NSAIDs [7]. Some NSAIDs cause gastrointestinal and renal adverse reactions because of their inhibitory effect on COX-1, while their therapeutic effects are mainly from the inhibitory activity on COX-2 [6]. Thus, a search for new anti-inflammatory substances with fewer side effects is necessary, especially for those substances that can be administered by topical application for local effect to avoid systemic adverse reactions.

Piperine-rich herbal mixture (PHM) used in this study is a traditional Thai medicine called "Sahastara remedy". It is used as an alternative medicine for muscle and bone pains and has been officially included in the Thai National List of Essential Medicine since A.D. 2011. The formulation of PHM contains the fruits of the black pepper (*Piper nigrum*) as a major constituent and also the long pepper (*Piper retrofractum*) fruits as shown in Table 1. Therefore, the anti-inflammatory activities of PHM mostly come from the bioactivities of piperine, which consists of these pepper fruits [8]. It has been reported that piperine inhibited the production of nitric oxide (NO), prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF-α) in lipopolysaccharide (LPS)-activated RAW264.7 cells. As an inhibitor, it could thereby suppress inducible NO synthase (iNOS), COX-2 and TNF-α gene and protein expression [9]. Pinsornsak et al. [10] investigated the efficacy of oral PHM for treatment of knee OA. The results showed that oral PHM reduced pain and improved ambulatory ability in patients who received orally 1,000 mg of PHM capsules 3 times per day for 28 days. Unfortunately, PHM sometimes caused heartburn and urticaria because of its high content of black pepper, as well as hepatic and renal toxicity from accumulation of camphor [10]. Consequently, alternative administration methods of PHM such as transdermal drug delivery could be considered as one of suitable administration methods to avoid the severe systemic side effects of PHM.

Film-forming solution (FFS) is a new product that is used for transdermal delivery of many drugs. Generally, FFS consists of drug(s), film-forming polymer(s) and necessary excipients, which are dissolved in a vehicle. FFS possesses fluidity characteristics, it thus can be sprayed or applied on skin easily. Upon evaporation of the vehicle, films will form on the skin and act as a matrix for sustained release of drug to the skin [11, 12, 13]. FFS is currently accepted as one of attractive transdermal drug delivery systems [12]. It has favorable properties and advantages over the conventional transdermal drug delivery systems in many aspects, i.e., wearing off resistance, presence of an invisible film on the skin, convenience of use by either spraying or applying on the lesion area, and causing less fixing of the skin.

Nowadays, use of traditional medicines has been promoted in conserving folk wisdom for healthcare. However, the actual efficacy and possible toxicity of some traditional medicines are still in doubt. Furthermore, they are usually inconvenient to be administered, for example, high doses with high frequencies of administration, unpleasant odors, unacceptable appearances and textures of the products themselves. Promoting the wide use of traditional medicines particularly among members of the younger generation will require consideration of the pharmacological effects of the remedies, along with the proper dosage form and the frequency of administration. Thus, the objectives of this study were to investigate anti-inflammatory activity of an alcoholic extract of PHM (PHM-E) and to evaluate physicochemical properties as well as anti-inflammatory activity of FFS containing PHM-E (PHM-E FFS).

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Dimethyl sulfoxide (DMSO), hydrochloric acid, sodium bicarbonate and sodium hydroxide were purchased from Univar, Australia. Ammonio methacrylate copolymer type A (Eudragit RL PO) and hydroxypropylcellulose (Klucel LF) were kindly provided by Darmstadt Ltd. (Germany) and Wilmington Ltd. (USA), respectively. All cell culture reagents were supplied by Gibco, USA. Cellulose dialysis tube with a molecular weight cutoff of 12,000, lipopolysaccharide (LPS) from *Escherichia coli*, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), N-(1-Naphthyl) ethylenediamine dihydrochloride, polyethylene glycol 400 (PEG 400) and standard piperine were obtained from Sigma-Aldrich, USA. All other chemicals and solvents were analytical grade and were used as received.

Table 1. Formulation of PHM [10].

Ingredients-Scientific name of the plants	Part of the used plants	Content (g)	Voucher specimen
1. *Acorus calamus*	Rhizome	8.8	SKP015010301
2. *Atractylodes lancea*	Rhizome	0.5	SKP051011201
3. *Anacyclus pyrethrum*	Root	0.6	SKP051011601
4. *Baliospermum montanum*	Root	8.0	SKP121021301
5. *Kleinhovia hospita*	Root	4.8	SKP183110801
6. *Merremia vitifolia*	Root	0.8	SKP054132201
7. *Picrorhiza kurroa*	Root	0.4	SKP177161101
8. *Plumbago indica*	Root	22.4	SKP148160901
9. *Anethum graveolens*	Fruit	1.0	SKP199010701
10. *Cuminum cyminum*	Fruit	0.8	SKP199030301
11. *Piper nigrum*	Fruit	24.0	SKP146161401
12. *Piper retrofractum*	Fruit	9.6	SKP146160301
13. *Terminalia chebula*	Fruit	10.4	SKP049200301
14. *Lepidium sativum*	Seed	1.1	SKP057121901
15. *Myristica fragrans*	Seed	1.2	SKP121130601
16. *Nigella sativa*	Seed	0.7	SKP160141901
17. *Pimpinella anisum*	Seed	0.9	SKP199160101
18. *Terminalia chebula*	Gall	0.3	SKP019200301
19. *Ferula assafoetida*	Oleo gum resin	0.1	SKP199060101
20. *Myristica fragrans*	Seed aril	1.3	SKP121130601
21. Camphor (powder)	-	1.4	SKP096030301

2.1.2. Plant materials

All ingredients of PHM listed in Table 1 were purchased from local traditional medicine suppliers. They were identified and authenticated by Center of Excellence on Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University, Thailand. Their specimen voucher number was assigned by the Herbarium of Southern Center of Thai Traditional Medicine, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkla, Thailand and also presented in Table 1.

2.2. Methods

2.2.1. Preparation of PHM-E

All ingredients in PHM formulation were weighed and mixed thoroughly. The mixture was macerated with 95% ethanol for three days to obtain PHM-E and then dried by use of an evaporator with a reduced pressure at 45 °C (Rotavapor R-205, Germany). The obtained PHM-E was chemically analyzed for piperine as a marker.

2.2.2. Determination of piperine content

Content of piperine in PHM-E and all samples was determined by using the high performance liquid chromatography (HPLC) technique. The analysis was performed by using the HPLC system (Agilent® 1200, USA) with a C18 column (Phenomenex® Luna, 4.6 × 250 mm, 5 micron). Ten µl of the samples were injected into the HPLC system and then eluted by gradient mobile phases composed of water/acetonitrile at various ratios as follows: 0 min, 60/40; 30 min, 50/50; and 60 min, 0/100 at a flow rate of 1.0 ml/min. The diode array detector was set at a wavelength of 340 nm.

2.2.3. Fourier-transform infrared (FT-IR) spectroscopy analysis

FT-IR spectroscopy analysis of standard piperine, PHM-E and obtained films from FFS with- and without PHM-E was carried out by using an FT-IR Spectrometer (PerkinElmer model Spectrum One, USA). Signal averages were obtained for 32 scans at a resolution of 4 cm^{-1} from 4,000 to 500 cm^{-1}.

2.2.4. Determination of in vitro anti-inflammatory activities

2.2.4.1. Effect of PHM-E on inhibition of nitric oxide (NO) production by the RAW 264.7 cells. The RAW 264.7 cells (ATCC, USA) were seeded in 96-well plates with a density of 10^4 cells/well containing 100 μl of a complete RPMI medium and were incubated until reaching a confluence (5% CO_2, 37 °C). The medium was then removed and replaced with 100 μl of fresh medium containing 4 ng/ml of lipopolysaccharide (LPS). Subsequently, 100 μl of PHM-E at various concentrations were added to each well, producing final concentrations of 0.1, 1, 10, 30 and 50 μg/ml. The cells were incubated for 24 h. Finally, the medium in each well was transferred to a new 96-well plate with the addition of the Griess reagent to detect nitrite (NO_2^-), which is a stable and non-volatile breakdown product of NO. The optical density (OD) of the medium was determined by using a microplate reader (Molecular Devices, USA) at a wavelength of 570 nm. Percentage of inhibition was calculated by using Eq. (1) and IC_{50} values were determined.

$$\text{Inhibition}(\%) = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100 \quad (1)$$

$OD_{control}$ and OD_{sample} were the OD of the following media: the media from the cells incubated with only LPS and the media from the cells incubated with LPS and the samples, respectively.

2.2.4.2. Effect of PHM-E on inhibition of prostaglandin E_2 (PGE_2) production by the RAW 264.7 cells. This study was performed by using a PGE_2 ELISA Kit (Cayman Chemical, USA). Briefly, after the RAW 264.7 cells were stimulated by LPS and incubated with PHM-E, supernatant (50 μl) was transferred to a 96-well pre-coated with mouse anti-rabbit IgG. Then, PGE_2 acetylcholinesterase tracer (50 μl) and PGE_2 monoclonal antibody (50 μl) were added into each well. They were incubated for 18 h at 4 °C without light exposure. The wells were then emptied and rinsed with a washing buffer. Thereafter, 200 μl of the Ellman's reagent were added to each well, and the plate was covered with plastic film and incubated in the dark for 1.5 h at 37 °C. The OD was read at a wavelength 412 nm by the microplate reader. The inhibition of PGE_2 production was calculated by using Eq. (2), and IC_{50} values were determined.

$$\text{Inhibition}(\%) = \frac{OD_{sample} - OD_{activated}}{OD_{control} - OD_{activated}} \times 100 \quad (2)$$

OD_{sample}, $OD_{activated}$ and $OD_{control}$ were the OD of the following media: the media from the cells incubated with LPS and the samples, the media from the cells incubated with only LPS, and the media from the cells without incubation with both LPS and the samples, respectively.

2.2.4.3. Effect of PHM-E on polarization of the RAW 264.7 cells. The activities of PHM-E in alteration of macrophage phenotype were studied in the RAW 264.7 cells following the previous publication by Chen et al. [14] with some modifications. The RAW 264.7 cells (M0) were incubated with either a mixture of LPS (100 ng/ml) and IFNγ (2.5 ng/ml) (Abcam, UK) or IL-4 (10 ng/ml) (Abcam, UK) for 12 h to induce M1 and M2 phenotype, respectively. The content of M1 and M2 phenotype markers in the cell culture medium, i.e. IL-1β, and IL-10, respectively, were determined by ELISA test kits for the rat cytokines (Abcam, UK). They were performed following the product protocols. Moreover, the content of the cytokines were also determined after these M1 and M2 cells were further incubated with PHM-E (14 μg/ml) for 12 h.

The results of this study were reported as fold changes of cytokine content in the medium. It was a ratio of the cytokine content released from the stimulated RAW 264.7 cells to the cytokine content released from the control, which was the untreated RAW 264.7 cells.

2.2.5. Determination of solubility of PHM-E in 65 % ethanol

The solubility of PHM-E in 65% ethanol, which was a vehicle of FFS, was investigated as follows: one ml of 65% ethanol was added to 1 g of PHM-E contained in an Eppendorf tube. The mixture was continuously shaken for 24 h at 37 °C and was centrifuged at 40,000 rpm for 30 min. The supernatant was filtered through a nylon filter (0.45 μm) and was then analyzed for the piperine content by the HPLC technique.

2.2.6. Preparation of FFS

FFS with PHM-E (PHM-E FFS) and FFS without PHM-E (Base FFS) containing Klucel LF, Eudragit RL PO and PEG 400 at a weight ratio of 4:4:1, respectively, were prepared. Briefly, Eudragit RL PO and Klucel LF were dispersed in 65% w/w ethanol and stirred continuously until clear solutions were obtained. PEG 400 was then added and stirred thoroughly. In the case of PHM-E FFS, PHM-E was added to Base FFS to make a final concentration of 3%w/w. The pH value of preparations was determined in triplicate by using a pH meter (Hanna instruments 8417, USA).

2.2.7. Rheological evaluation of base FFS and PHM-E FFS

The rheological evaluation of Base FFS and PHM-E FFS was performed in triplicate at 25 ± 0.1 °C using a controlled stress rheometer (Bohlin Gemini HR nano, Malvern instrument, UK) with a cone and plate geometry (2° cone and 55 mm diameter). Shear stress and shear viscosity of the samples were determined by a steady shear sweep mode with initial and final shear rates set at 0.1 and 1,000 s^{-1}, respectively.

2.2.8. Determination of mechanical properties of the films obtained from base FFS and PHM-E FFS

Dry films of Base FFS and PHM-E FFS, which were called briefly as "Base film" and "PHM-E film", respectively, were prepared as follows: Base FFS and PHM-E FFS (24 g) were poured onto Teflon molds (6 × 8 cm) and then dried in a hot-air oven at temperature of 45 °C (Memmert, Germany) for 12 h.

To determine their mechanical properties, the films were cut into a rectangular shape (15 × 80 mm) and measured the tensile strength and % elongation at break by using a Universal Testing Machine (Instron 8801, USA) equipped with a 10 N load. The films were placed between two vertical grips, which were set at an initial distance of 40 mm. The movable grip was then moved upward at a speed of 500 mm/min until the films were rupture [13].

Tackiness of Base film and PHM-E film (2 × 2 cm) were determined by a probe tack test using a controlled-strain rheometer (model ARES-G2, TA Instrument, USA). The instrument had a normal force-measuring unit equipped with a probe tack fixture (5 mm diameter). An axial force compression at 10 Pa-constant stress with 1 s-soak time duration, 0.01 mm-maximum gap change and 1 point/s-sampling rate were set. The tacking probe was moving away from the samples' surface at a speed of 10 mm/s with 5 s-soak time duration.

2.2.9. In vitro release study of PHM-E film

Release of piperine from PHM-E films through a cellulose dialysis membrane was determined by using modified Franz diffusion cells following the previous publication [13]. Briefly, PHM-E films were cut into 2 × 2 cm for a proper covering of the diffusion area (1.78 cm^2) and were then placed onto the cellulose dialysis membrane. The membrane with PHM-E film faced to a donor unit was placed between the donor and receptor units, which were filled with ethanol (10% v/v) in phosphate buffered saline (PBS) (pH 7.4) under continuously stirring by a magnetic stirrer and maintenance at 37 ± 1 °C. The receiving solutions were withdrawn at 12 time-points (5, 10, 15, 30, 60, 120, 180, 240, 300, 360,

420 and 480 min) and timely replaced with same volume of fresh media. Finally, the withdrawn receiving solutions were analyzed for piperine content by the HPLC technique.

2.2.10. In vitro skin permeation study of PHM-E film

Skin permeation of piperine from PHM-E films was investigated in triplicate by using modified Franz diffusion cells and the skin from newborn pigs those died naturally after birth. The skin was supplied by a local pig farm. It was removed subcutaneous fat and then cut into 2 × 2 cm before storage at -18 °C. One hour prior to the experiment, the skin was equilibrated in PBS (pH 7.4) at 37 °C [15]. PHM-E films with the size of 2 × 2 cm, which covered the diffusion area (1.78 cm^2), were placed on the epidermis of the skin and faced to the donor unit. Then, they were put on the receptor unit containing ethanol (10% v/v) in PBS (pH 7.4), which was a receiving solution and was under stirring by a magnetic stirrer with temperature control at 37 ± 1 °C. The receiving solutions were withdrawn at 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 24 h. Thereafter, the fresh solutions were replaced to maintain the constant volume of the receiving solution. The piperine content in the receiving solutions was determined by the HPLC technique. The flux of piperine through the skin was calculated according to Fick's first law of diffusion [15].

2.2.11. Skin toxicity of PHM-E FFS

In this study, Base FFS, PHM-E FFS and their films were tested for determination of their toxicity to skin fibroblasts. Each sample was dispersed in a complete DMEM medium to make sample dispersions with various concentrations of 2, 20, 60 and 100 μg/ml.

Human Dermal Fibroblasts, neonatal (HDFn) cells (Thermo Fisher Scientific, USA) were cultured in a complete DMEM and seeded in 96-well plates with a density of 10^4 cells/well/100 μl and incubated for 24 h in a CO_2 incubator at 37 °C. Thereafter, 100 μl of sample solutions at various concentrations were added to the wells, giving final concentration of 1, 10, 30, and 50 μg/ml. The cells were incubated with the sample solutions for 24 h and washed twice with PBS (pH 7.4) after the end of incubation period. Fifty μl of MTT (0.5 mg/ml) in the medium were added to each well and incubated for four hours. The medium was then removed, and DMSO was added to dissolve the formazan crystal (100 μl/well). The OD of each well was measured at a wavelength of 570 nm by the microplate reader. Percentage of cell viability was calculated using Eq. (3). The test samples were considered to be cytotoxic if the cell viability was less than 70% [16].

$$\text{Cell viability } (\%) = \frac{OD_{sample}}{OD_{control}} \times 100 \quad (3)$$

OD_{sample} and $OD_{control}$ were the OD of the following media: the media from the HDFn cells incubated with the samples and MTT solution, and the media from the HDFn cells incubated with only MTT solution, respectively.

2.2.12. Eye irritation test of PHM-E FFS

Due to fluidity properties of PHM-E FFS, this product could be sprayed or applied on the skin for pain relief easily. Meanwhile, it might be accidentally spilled and splashed into the eye. Therefore, the eye irritation potential of PHM-E FFS had to be evaluated. In this study, the short time exposure (STE) test was performed following the OECD guideline [17]. Briefly, SIRC cells (the rabbit corneal cell line) (ATCC, USA) were cultured in complete MEM and seeded in 96-well plates with a density of 10^4 cells/well/100 μl and incubated in a CO_2 incubator at 37 °C until reaching a confluence. Then, they were exposed to 200 μl of either 5% or 0.05% w/v PHM-E FFS dissolved in normal saline for 5 min. Thereafter, the cells were washed twice with PBS (pH 7.4). Two hundred microliters of MTT (0.5 mg/ml) in the medium were added to each well and incubated for two hours. The medium was then removed, and 0.04 N hydrochloric acid-isopropanol was added to dissolve the formazan crystal (200 μl/well) for an hour. The OD of each well was measured at a wavelength of 570 nm by the microplate reader. For this study, the 100% cell viability (CV) was calculated from the OD of the wells containing the SIRC cells without exposure to the test solutions. The result was shown as a mean of CV ± SD.

The eye irritation potential from the test was scored following the criteria of the STE test. The summed scores were used to rank the eye irritation potential as follows: the total score of 1, 2 and 3 were defined as the minimal ocular irritant, the moderate ocular irritant and the severe ocular irritant, respectively.

2.2.13. In vivo anti-inflammatory test of PHM-E FFS

Eighteen male Wistar rats weighing 120 ± 10 g were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The procedure for use and care of the animals for this study was approved by the Animal Care Committee at the Thailand Institute of Scientific and Technological Research (TISTR) (Project code #PS-59002). The animal experiment was conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, in accordance with the spirit of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) for international's expectations of animal care and use/ethics committees.

Before starting the test for 7 days, a preliminary experiment was performed to determine the potential of Base FFS, phenylbutazone, PHM-E FFS and 5% w/v ethyl 3-phenylpropiolate (EPP) (Sigma-Aldrich, USA) solution in acetone, for induction of skin irritation by applying these test samples on the rats' inner and outer left ear surface (10 μl each) (n = 3). The signs of skin irritation and inflammation, i.e. skin rash, erythema and edema of the rats' ears were observed at day 1, 2 and 3 after application.

The *in vivo* anti-inflammatory test was modified based on the previous report by Dunstan et al. [18]. The rats were equally divided into three groups (6 rats/group) as follows: 1) a negative control group, 2) a positive control group and 3) a test group. They received Base FFS, phenylbutazone and PHM-E FFS dissolved in acetone, respectively. The thickness of the rats' right ear was measured before starting the test by a pocket thickness gauge (Mitutoyo, Japan) for use at the baseline of ear thickness. Each test samples diluted with acetone was applied onto the inner and outer right ear surface (10 μl each). Thirty minutes later, the right ear of each rat was treated with 5% w/v ethyl EPP in acetone by application on both surfaces of each ear. Thereafter, the thicknesses of the ears were measured at 30 min, 1 h and 2 h after the inductions. The percentage of edema inhibition was calculated at the indicated time intervals.

At the end of the study, the rats were immediately euthanized and the treated ears were collected. The rats' ears were immersed in formalin solution (10% v/v in PBS) (Sigma-Aldrich, USA), paraffin-embedded, sliced and hematoxylin and eosin (H&E) stained [1]. The stained rats' ear tissues were then observed under a light microscope (Nikon Eclipse E200, Japan) for comparison of thickness as well as cell infiltration in the rats' ear tissue.

2.2.14. Determination of IL-1β and TNF-α content in the rats' ear tissue

To determine the cytokine content in the treated rats' ear, content of IL-1β and TNF-α in the rats' ear tissue were measured [19]. Each tissue sample (0.05 g) from the rats' ear that received PHM-E FFS, Base FFS or phenylbutazone and exposed to EPP for 2 h were homogenized in PBS (pH 7.4) containing sodium chloride (0.4 mol/l), Tween 20 (0.05% w/v), bovine serum albumin (0.5% v/v), benzethonium chloride (0.1 mmol/l), EDTA (10 mmol/l) and aprotinin (20 KIU/ml). The lysates were centrifuged at 10,000 rpm at 4 °C for 60 min. Thereafter, the supernatant was collected for analysis of IL-1β and TNF-α by using ELISA kits for the rat IL-1β and TNF-α, respectively (Abcam, UK). They were performed following the product protocols.

2.2.15. Statistical analysis

Experimental results were presented as a mean with either standard deviation (SD) or standard error of mean (SEM). Statistical analysis for

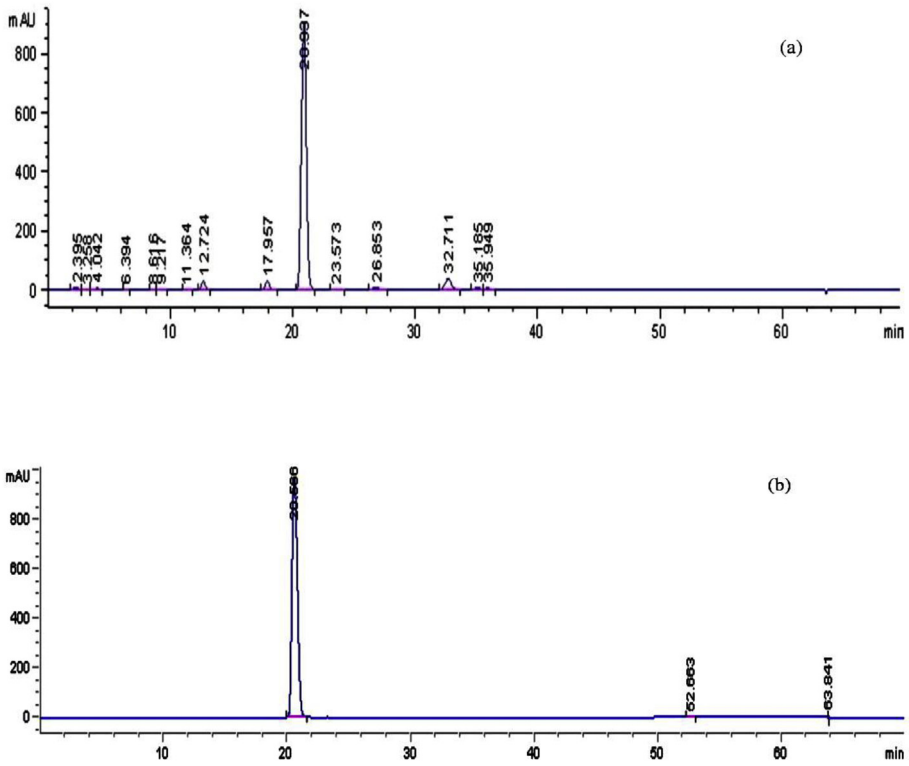


Figure. 1. Chromatograms: (a) piperine in PHM-E and (b) standard piperine.

comparing treatment effects were performed by either an independent T-test or a one-way analysis of variance (ANOVA) with Tukey's HSD Post Hoc Test at a significant level of 0.05.

3. Results and discussion

3.1. Preparation and characterization of PHM-E

The obtained PHM-E was a clear solution with a slightly dark green color and a characteristic odor. The calculated percentage yield of PHM-E was 38.4% w/w as based on total weight of dry PHM. The pH value of PHM-E was 5.41 ± 0.00 suggesting that it was compatible with the pH value of a normal skin surface (which is approximately 5.00) [20,21].

HPLC chromatograms of PHM-E and standard piperine are shown in Figure 1 (a) and (b). The chromatogram peak of the extract appeared at the same retention time of standard piperine, which was around 20 min. The timing of this peak indicated that the major constituent of the extract was piperine as derived mainly from the fruits of black pepper (*Piper nigrum*) and long pepper (*Piper retrofractum*). A linear regression equation for the determination of piperine content is presented in Eq. (4)

$$\text{Area under curve}_{\text{piperine}} = 72.38(\text{Concentration}_{\text{piperine}}) + 203.2;\ r^2 = 0.9998 \quad (4)$$

The content of piperine in PHM-E as determined by the HPLC technique and calculated by use of Eq. (4) was 85.08 ± 1.03 mg/g. This finding suggested that the extraction procedure used in this study could efficiently extract piperine from the remedy to obtain PHM-E containing 8.51% w/w of piperine.

Figure 2 (a) exhibited major IR absorption bands of standard piperine at wavenumbers of 1582, 1633, 2940 and 3008 cm^{-1} corresponding to ketone, amine, CH_2–CH_2–CH_3, and alkene groups in the piperine molecule, respectively. They were in consistent with the FT-IR spectrum of piperine presented in previous reports [22, 23]. The FT-IR spectrum of PHM-E illustrated in Figure 2 (b) also showed IR absorption bands of the amine- and CH_2–CH_2–CH_3 group of piperine with trivial shifts to wavenumbers of 1632 and 2938 cm^{-1}, respectively. However, the characteristic bands of ketone and alkene groups found in FT-IR spectrum of standard piperine could not be observed in this spectrum. This finding could be explained by interactions between piperine and other compounds in PHM-E.

3.2. Determination of in vitro anti-inflammatories activity of PHM-E

3.2.1. Effect of PHM-E on inhibition of NO and PGE_2 production by the RAW 264.7 cells

The results shown in Figure 3 (a) revealed that the higher the concentration of PHM-E, the higher the percentage inhibition of both NO and PGE_2 production. These findings were in agreement with the previous report by Bang et al. [24]. They found that anti-inflammatory activity of piperine was dependent on the doses being administered. Consequently, anti-inflammatory activities of PHM-E increased in a dose-dependent manner. Furthermore, the IC_{50} values of PHM-E for inhibition of NO and PGE2 production were 7.92 ± 0.23 and 13.23 ± 0.23 μg/ml, respectively.

3.2.2. Effect of PHM-E on polarization of the RAW 264.7 cells

In this study, the effect of PHM-E on phenotype polarization of the RAW 264.7 cells was investigated. The obtained results are shown in Figure 3(b). It presents the fold change of the released cytokine content that was a ratio of the released cytokine content from the stimulated cells to that of the control. It was found that the RAW 264.7 cells treated with the mixture of LPS and IFNγ released higher IL-1β content than that of the control (p-value = 0.000), while, the released content of IL-10 were comparable to that of the control (p-value = 0.952). This result suggested that the RAW 264.7 cells phenotype was polarized from M0 to M1 by the mixture of LPS and IFNγ. The data presented in Figure 3 (b) also pointed out that the phenotype of the RAW 267.4 cells was changed from M0 to M2 by stimulation of the cells with IL-4. It showed that the IL-4-treated RAW 264.7 cells released IL-1β at the similar content to that of the control (p-value = 0.999). However, they released higher content of IL-10 than that of the control (p-value = 0.000) [14].

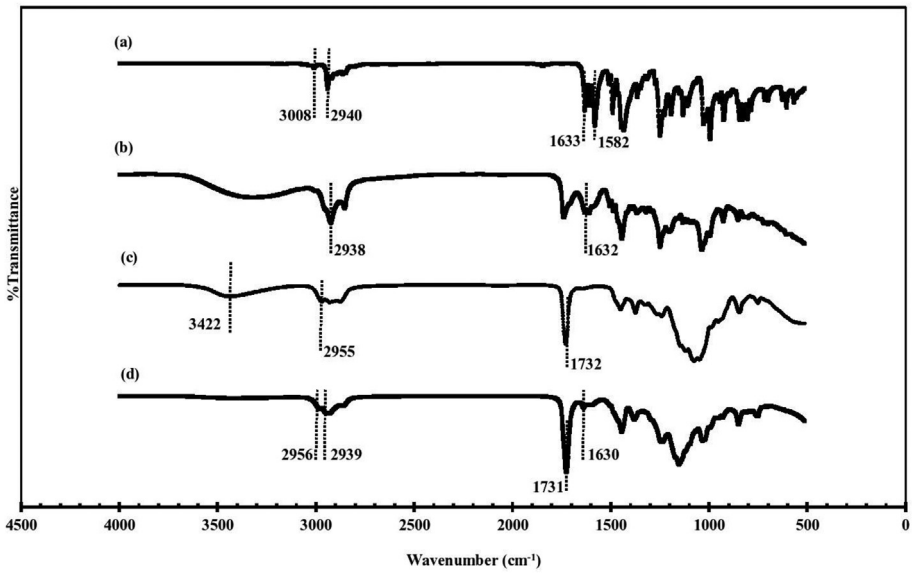
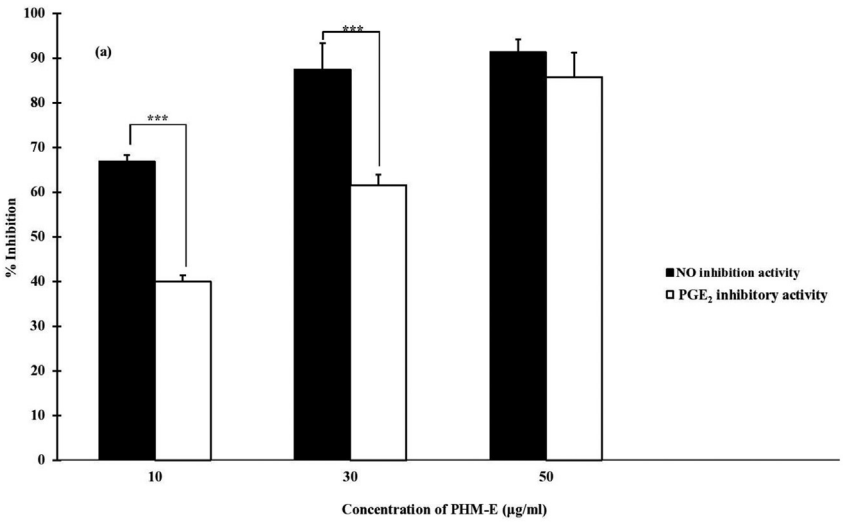


Figure 2. FT-IR spectra: (a) standard piperine; (b) PHM-E; (c) base film; (d) PHM-E film.



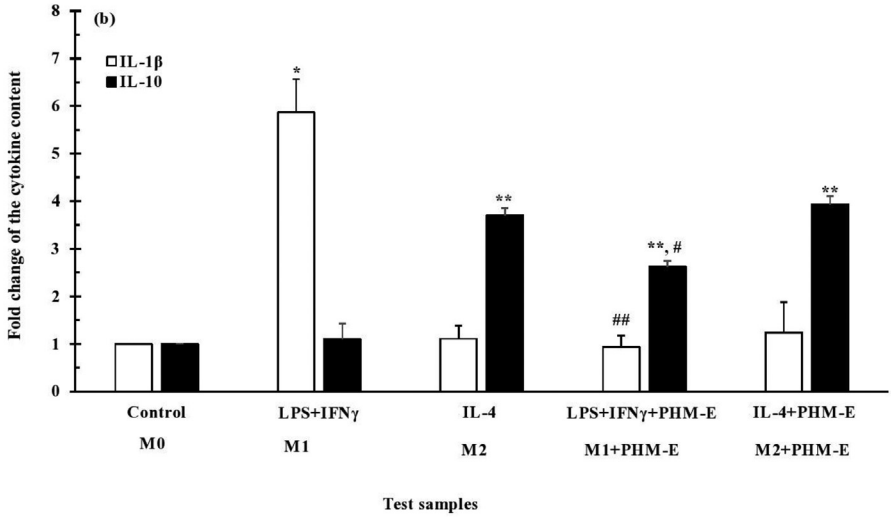


Figure 3. Anti-inflammatory activities of PHM-E (mean ± SEM; n = 3): (a) percentage of inhibitory effect of PHM-E on NO and PGE_2 production (*** significantly different at p-value < 0.05); (b) Fold change of IL-1β and IL-10 after incubation of the RAW 264.7 cells with the test samples (*, ** significantly different from each cytokine content of the control (M0) at p-value < 0.05; #, ## significantly different from each cytokine content of the cells treated with a mixture of LPS and IFNγ without PHM-E (M1) at p-value <0.05).

Figure 3(b) indicated that IL-10 content from the medium of the cells incubated with the mixture of LPS and IFNγ and further incubated with PHM-E (M1+PHM-E) was significantly higher than that of the control and the cells incubated with the mixture of LPS and IFNγ without PHM-E (M1) (p-value = 0.000 and 0.000, respectively). Meanwhile, the content of IL-1β were dramatically decreased to the similar content to that of the control (M0) (p-value = 1.000) and were lower than that of the cells incubated with the mixture of LPS and IFNγ without PHM-E (M1) (p-value = 0.000). This finding evidenced that the RAW 264.7 cells phenotype was polarized from M1 to M2 by PHM-E.

The IL-10 content in the medium of the RAW 264.7 cells those were treated with IL-4 and PHM-E (M2+PHM-E) were comparable to that of the cells treated with IL-4 (M2) only (p-value = 0.511) and were higher than that of the control (M0) (p-value = 0.000). The IL-1β content from these cells were not significantly different with that of the cells treated with IL-4 only (M2) and the control (M0) at p-values of 0.969 and 0.996, respectively. The results demonstrated that the M2 phenotype of the RAW 264.7 cells was not changed to M1 by PHM-E. Therefore, PHM-E could be accepted as an anti-inflammatory agent acting via the inhibition of NO and PGE_2 production by macrophages and promotion of macrophage phenotype polarization from M1 to M2.

The obtained results were consistent with the previous study [25]. It reported that PHM-E could inhibit production of IL-1β, which regulates the activity of other pro-inflammatory mediators of inflammatory process in the primary human dermal fibroblasts. PHM-E thus had potential for using as an alternative substance in treatment of inflammatory diseases in various organs which include muscle pain, rheumatoid arthritis, osteoarthritis and ankylosing spondylitis [26, 27, 28, 29, 30].

3.3. Determination of solubility of PHM-E in 65% ethanol

Solubility of PHM-E in 65% ethanol, which was a vehicle of PHM-E FFS, was investigated to determine the maximum content of PHM-E that could be loaded into the formulation. It was found that solubility of PHM-E in 65% ethanol was 0.37 ± 0.00 g/ml (mean ± SD; n = 3) equivalent to the piperine content of 31.6 ± 0.1 mg/ml (mean ± SD; n = 3). It could be concluded that PHM-E was freely soluble in 65% ethanol.

3.4. Preparation and characterization of base FFS and PHM-E FFS

Base FFS and PHM-E FFS prepared in this study were homogeneous solutions without precipitation of any ingredients. The obtained Base FFS was clear in appearance and colorless. After PHM-E was added to Base FFS, the clear, brown PHM-E FFS with the mild characteristic odor of PHM-E was obtained. PHM-E FFS had a pH value of 5.98 ± 0.03 which differed from the pH 6.74 ± 0.04 (p-value = 0.000) of Base FFS since the acidity of PHM-E remained as reported in the previous section. However, this pH value was still in the range of pH values that are generally known to be safe for transdermal applications.

3.5. Rheological evaluation of base FFS and PHM-E FFS

The study of rheological properties showed the linear relationship between shear stress and shear rate of Base FFS and PHM-E FFS, which are represented by the Eqs. (5) and (6), respectively.

$$\text{Shear stress}_{\text{Base FFS}} = 0.087 \text{ x Shear rate}_{\text{Base FFS}};\ r^2 = 0.9998 \quad (5)$$

$$\text{Shear stress}_{\text{PHM-E FFS}} = 0.056 \text{ x Shear rate}_{\text{PHM-E FFS}};\ r^2 = 0.9997 \quad (6)$$

The results indicated that Base FFS and PHM-E FFS had the Newtonian flow behavior with a low viscosity of 0.087 ± 0.003 and 0.056 ± 0.004 Pas, respectively. They implied that both Base FFS and PHM-E FFS could be administered easily by either spraying or applying on skin. However, the lower viscosity of PHM-E FFS compared to Base FFS was observed (p-value = 0.000). This result indicated that PHM-E in PHM-E FFS decreased polymer chain-solvent interactions [31].

3.6. Characterization of base film and PHM-E film

The Figure 2 (c) showed important bands of Base film at wavenumbers 1732 and 2955 cm^{-1} that were assigned to the carbonyl ester vibration and the C–H stretching of Eudragit RL PO, respectively; and 3422 cm^{-1} corresponded to the hydroxyl group in Klucel LF. In PHM-E film, characteristic bands of piperine with minor shifts were found as shown in in Figure 2 (d); these shifts were observed at wavenumbers of 1630 and 2939 cm^{-1} representing amine- and CH_2–CH_2–CH_3 group in the piperine molecule, respectively. Although some important bands of film base still appeared at nearly the same wavenumber as previously shown in Figure 2 (c), the band of hydroxyl group in Klucel LF at wavenumber of 3422 cm^{-1} had disappeared. It is possible that intermolecular interactions such as H-bonding between the hydroxyl groups of film base and the piperine molecule had occurred.

The study of mechanical properties indicated that PHM-E film possessed an obviously lower tensile strength (0.99 ± 0.09 MPa) than that of Base film (4.04 ± 0.04 MPa) at a p-value of 0.000. It is possible that PHM-E reduced intermolecular forces between polymer chains and numbers of polymer-polymer contact leading to a decrease in the rigidity of the three-dimensional network in PHM-E film [32, 33, 34]. On the contrary, PHM-E film had significantly a greater percentage elongation at break (5.33 ± 0.12%) than that of Base film (3.13 ± 0.06%) at a p-value of 0.000. Thus, PHM-E had a plasticizing effect on the films [35]. The results from this study also showed that PHM-E film had a higher tackiness (18.05 ± 0.05 gf) than that of Base film (13.97 ± 0.05 gf) at a p-value of 0.000. It was due to the fact that PHM-E reduced the interactions of the polymer chain-chain causing the polymer chains to move freely, and then, interacted firmly with the test probe surface [33]. However, it did not cause an obviously sticky feel on the skin. Therefore, PHM-E FSS had advantages for the topical application of PHM-E because it provided PHM-E film having flexibility, extensibility and anti-peel-off properties.

3.7. In vitro release study of PHM-E film

The release profile of PHM-E film is illustrated in Figure 4 (a). During the first 15 min of the experiment, the piperine content could not be detected in the receiving solutions. It is possible that the films needed more time for properly moistening by the receiving solution [36]. Furthermore, the amount of piperine released from the films at the beginning of the study was less than the limit of detection of the analysis method.

The release profile of the PHM-E film in Figure 4 (b) showed that following the first 60 min, the piperine was released continuously to the receiving solutions consistent with a zero-order kinetic model as shown in Eq. (7) at a constant rate of 0.19 μg/min.

$$\text{Piperine released content} = 0.19(\text{Time}) - 11.07;\ r^2 = 0.9928 \quad (7)$$

This finding indicated that the release rate of piperine from PHM-E film was constant for the entire experiment and independent of any drug concentrations in the film. A steady release rate constant of the PHM-E film over time made possible the benefits of this PHM-E film. It could minimize the frequency of drug administration and lessen the side effects of PHM-E from reduction in its usage [37]. Therefore, FFS could be used as an effective carrier for topically controlled release of piperine from PHM-E.

3.8. In vitro skin permeation study of PHM-E film

The skin permeation profile of piperine from PHM-E film is presented in Figure 5 (a). It shows the non-steady state of skin permeation of piperine at the first 3 h of the experiment, and then the steady state of skin permeation of piperine as seen in the linear portion of the plot. The lag time of skin permeation of piperine, which was determined by

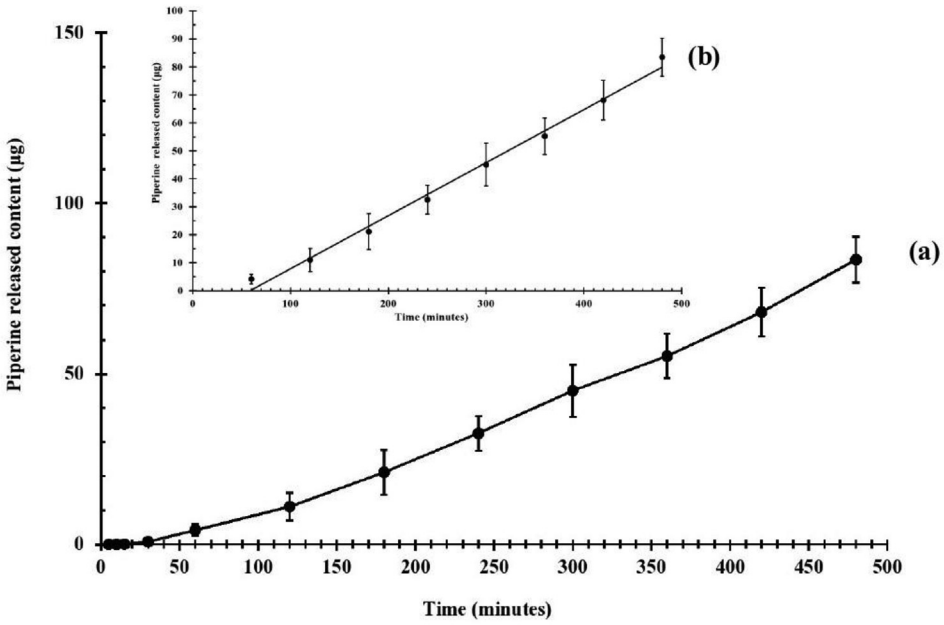


Figure 4. Release profile of piperine from PHM-E film (mean ± SD; n = 3): (a) piperine released content against time; (b) linear relationship between piperine released content and time during 60–480 min.

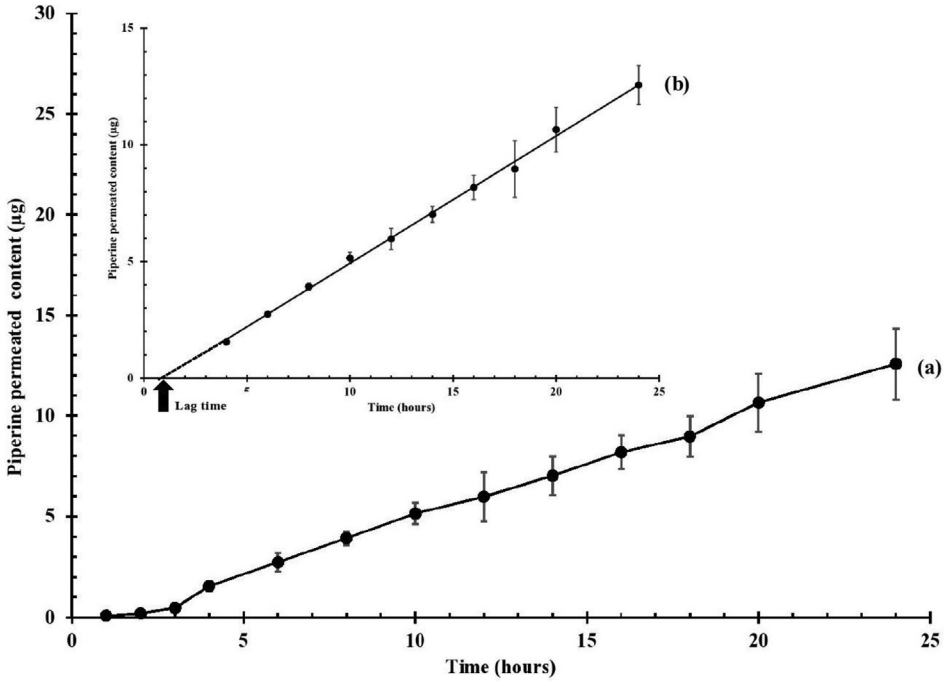


Figure 5. Skin permeation profile of piperine from PHM-E film (mean ± SD; n = 3): (a) piperine permeated content against time; (b) linear relationship between piperine permeated content and time during 4–24 h.

extrapolating the linear portion of the steady state permeation profile to the time axis (Figure 5 (b)), was 57.6 ± 0.5 min (0.96 ± 0.01 h). It indicated that the concentration gradient of piperine in the skin reached equilibrium at around 1 h after the beginning of the experiment.

The linear portion of the permeation profile shown in Figure 5 (b) was described by Eq. (8). It was used for determination of the flux of piperine according to Fick's first law of diffusion [15].

$$\text{Piperine permeated content} = 0.55(\text{Time}) - 0.52;\ r^2 = 0.9977 \quad (8)$$

It was found that the flux of piperine through the skin was 0.31 ± 0.04 µg/cm^2·hour. This result implied that approximately 0.31 µg of piperine from PHM-E film could permeate through a unit cross section of the skin in an hour.

3.9. Skin toxicity test of PHM-E FFS

The results of *in vitro* skin toxicity test of Base FFS, PHM-E FFS, Base film and PHM-E film on the HDFn cells are shown in Table 2. It indicated that the HDFn cells could survive after exposure to all test samples with the cell viability of around 100% which was more than 70%. The test samples, thus, were not toxic to the skin cells at entire test concentrations [38] and could be accepted as safe for further *in vivo* study.

Table 2. Cell viability of the HDFn cells exposed to test samples (mean ± SD, n = 3).

Test Sample	Cell viability (%) of the cells at various concentrations of test samples			
	1 μg/ml	10 μg/ml	30 μg/ml	50 μg/ml
FFS base	102.3 ± 2.2	103.2 ± 5.0	102.5 ± 2.2	102.6 ± 4.6
PHM-E FFS	100.6 ± 3.6	101.4 ± 3.8	99.7 ± 4.7	100.8 ± 5.0
Base film	100.3 ± 5.8	101.5 ± 3.8	101.7 ± 5.0	101.1 ± 6.0
PHM-E film	102.9 ± 5.1	101.3 ± 2.0	100.3 ± 5.0	100.2 ± 4.2

Table 3. Scores obtained from the short time exposure (STE) test of PHM-E FFS.

Concentration (w/v)	CV of SIRC cells (%)*	Criteria for scoring	Obtained scores
5%	98.4 ± 1.8	If CV > 70%: scored 0 If CV ≤ 70%: scored 1	0
0.05%	101.0 ± 0.2	If CV > 70%: scored 1 If CV ≤ 70%: scored 2	1
		Total score	1

* (mean ± SD, n = 6).

3.10. Eye irritation test of PHM-E FFS

As previously mentioned, PHM-E FFS had fluidity characteristics, it could be either sprayed or topically applied on the skin. However, this product might be unintentionally splashed into the eye, therefore, eye irritation potential of PHM-E FFS was determined following the suggestion by the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS) [17]. The CV of the SIRC cells after exposure to 5% and 0.05% w/v of PHM-E FFS for 5 min are shown in Table 3. The total score from summation of the obtained scores following the criteria of STE test was equal to 1. Therefore, PHM-E FFS was categorized as a minimal ocular irritant when it was accidentally spilled or splashed into the eye.

3.11. In vivo anti-inflammatory test of PHM-E FFS

The results of preliminary experiment indicated that Base FFS, phenylbutazone and PHM-E FFS dissolved in acetone did not cause symptoms of skin irritation and inflammation in all treated rats' ears during the 3-day experiment. Meanwhile, skin rash, erythema and edema were obviously observed in the rats' ears which exposed to EPP solution within 1 h after application. These results suggested that Base FFS, phenylbutazone and PHM-E FFS were safe to the rats' skin. On the other hand, EPP solution was a strong skin irritant that could induce skin inflammation in the rats' ear.

Figure 6 (A) shows that the rats receiving PHM-E FFS had a significantly lower ear thickness than that of the rats receiving Base FFS at *p*-values of 0.001, 0.000 and 0.006 after ear edema induction for 30 min, 1 h and 2 h, respectively. They also had an obviously lower ear thickness than that of the rats receiving phenylbutazone at *p*-values of 0.022, 0.01 and 0.002 at the same time intervals. Although the ear thickness of the rats' ear treated with phenylbutazone and Base FFS were different at *p*-values of 0.003 and 0.007 after ear edema induction for 30 min and 1 h, respectively, they were statistically comparable at 2 h after ear edema induction (*p*-value = 0.100). This result suggested that anti-inflammatory activity of phenylbutazone dramatically decreased within 2 h.

It was found that PHM-E FFS could inhibit rat ear edema by about 67.9%, 64.6% and 39.4% after ear edema induction for 30 min, 1 and 2 h, respectively. Meanwhile, phenylbutazone and Base FFS could inhibit rat ear edema at 30 min by about 43.6% and 18.6%; at 1 h by about 37.1% and 18.4%; as well as at 2 h by about 16.7% and 15.5%, respectively. Dunstan et al. [18] recommended that the anti-inflammatory activity of test samples could be categorized according to the following criteria: strong activity = more than 70% of rat ear edema inhibition; moderate activity = 40–69%; low activity = 20–39% and not active = less than 20%. Therefore, PHM-E FFS had a moderate-to-high anti-inflammatory activity, while, phenylbutazone, a standard substance used as a positive control for this study, had a moderate-to-low activity. However, Base FFS did not have anti-inflammatory activity. The results implied that

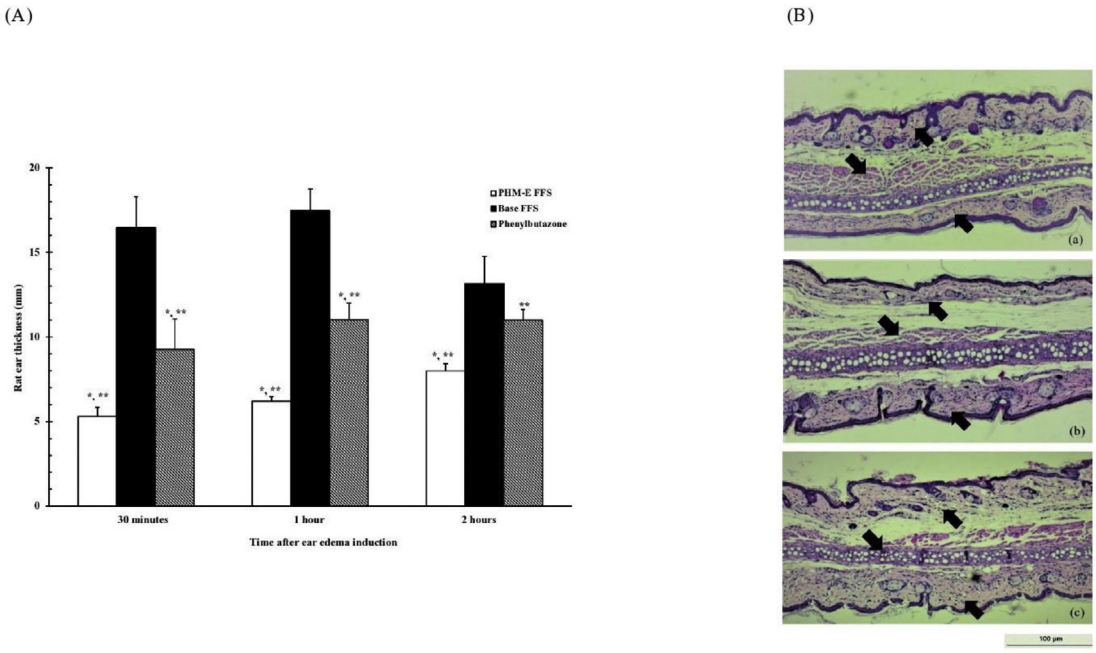


Figure 6. *In vivo* anti-inflammatory activity test: (A) Thickness of the rats' ear after treatment with the test samples and ear edema induction (mean ± SD; n = 6) (*significantly different from Base FFS, **significantly different of each other between PHM-E FFS and phenylbutazone, at *p*-value < 0.05). (B) Photographs of longitudinal section of the rats' ear with H&E staining after treatment with the test samples and ear edema induction for 2 h: (a) PHM-E FFS; (b) base FFS and (c) phenylbutazone.

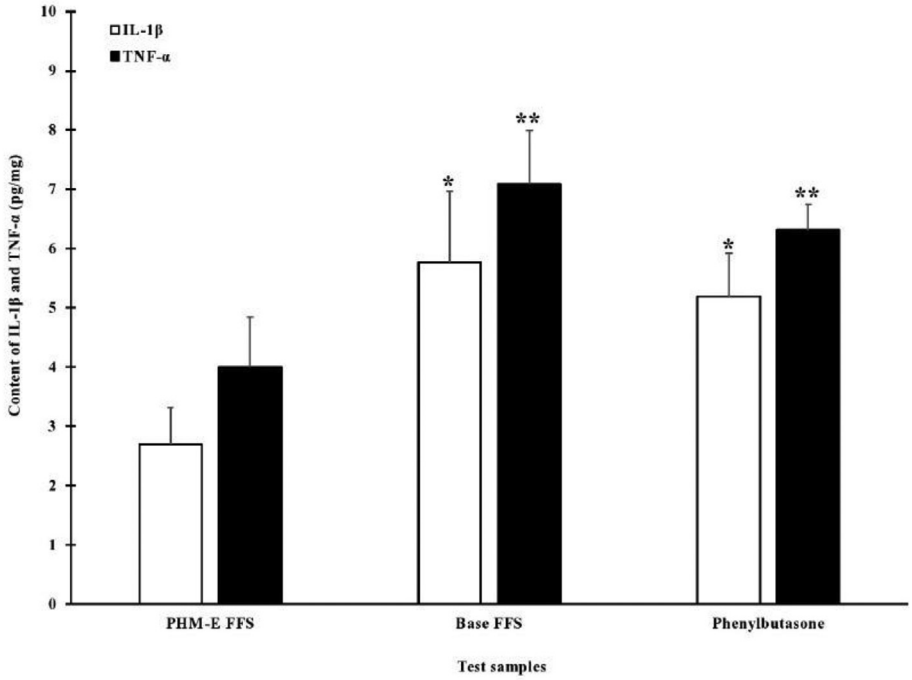


Figure 7. Content of IL-1β and TNF-α in the rats' ear tissue after treatment with the test samples and ear edema induction for 2 h (mean ± SD; n = 3) (*, **significantly different from each cytokine content of the rats' ear receiving PHM-E FFS at *p*-value < 0.05).

anti-inflammatory of PHM-E FFS was mainly from PHM-E consisting of the formulation.

These findings were confirmed by the photographs of H&E-stained rats' ear tissues after treatment with the test samples and ear edema induction for 2 h as illustrated in Figure 6 (B). Figure 6 (B)–(a) demonstrated that the rats' ear treated with PHM-E FFS had lower thickness and less cell filtration than those of the rats' ear treated with Base FFS and phenylbutazone as depicted in Figure 6 (B)–(b) and (c), respectively. It indicated that PHM-E FFS could effectively reduce ear edema and cell filtration, which was neutrophil-rich associated with edema [1].

3.12. Determination of IL-1β and TNF-α content in the rats'ear tissue

The content of IL-1β and TNF-α in the rats' ear tissue after treatment with the test samples and ear edema induction for 2 h are presented in Figure 7. The results showed that IL-1β content in the rats' ear receiving Base FFS and phenylbutasone were significantly higher than that of the rats' ear receiving PHM-E FFS at a *p*-value of 0.013 and 0.032, respectively. They were in agreement with the TNF-α content in the rats' ear treated with Base FFS and phenylbutasone, which were higher than that of the rats' ear exposed to PHM-E FFS at *p*-values of 0.006 and 0.022, respectively. These findings pointed out that PHM-E FFS could decrease the content of pro-inflammatory cytokines in the inflamed tissue, in particular, IL-1β and TNF-α and also evidenced that PHM-E FFS had a stronger anti-inflammatory activity than that of Base FFS and phenylbutazone. Therefore, it has become possible to use PHM-E FFS for delaying the progress of inflammatory diseases.

4. Conclusions

PHM-E obtained from this study showed anti-inflammatory activity via inhibition of NO and PGE_2 released from the RAW 264.7 cells and promotion of the cell phenotype polarization from M1 to M2. After incorporation of PHM-E into FFS, physicochemical properties of the obtained PHM-E FFS, i.e. its physical appearance, pH, and viscosity were different from those of Base FFS. It was found that PHM-E film had a lower tensile strength, a greater percentage elongation at break and a greater tackiness than those of the Base film. The release of PHM-E from PHM-E FFS films was in agreement with a zero-order kinetic model. The skin toxicity test indicated that Base FFS, PHM-E FFS, Base film and PHM-E film could be accepted as safe for topical use. The result of eye irritation test suggested that PHM-E FFS could be categorized as a minimal ocular irritant when it was accidentally spilled or splashed into the eye. From the *in vivo* anti-inflammatory activity test, PHM-E FFS could reduce the rats' ear edema with a higher percentage inhibitory activity than Base FFS and phenylbutazone. PHM-E FFS was thus classified as a moderate-to-high anti-inflammatory product and had potential for being used in a clinical study to investigate its efficacy and safety in patients.

Declarations

Author contribution statement

R. Asasutjarit: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

P. Sookdee: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

S. Veeranondha: Performed the experiments; Analyzed and interpreted the data.

A. Fuongfuchat: Analyzed and interpreted the data; Wrote the paper.

A. Itharat: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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