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School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Development and evaluation of Sahasthara Thai medicine remedy in a film-forming spray for topical anti-inflammatory therapy

Ninnart Intharit[1], Arunporn Itharat[2,*], Chadchom Choockong[2], Weerachai Pipatrattanaseree[3], Wichan Ketjinda[4], Neal M. Davies[5], and Raimar Löbenberg[5]

[1]Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand.
[2]Department of Applied Thai Traditional Medicine and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University, Khlong Luang, Pathum Thani, 12120, Thailand.
[3]Regional Medical Science Center 12 Songkhla, Department of Medical Sciences, Songkhla 90100, Thailand.
[4]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Songkhla 90112, Thailand.
[5]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB T6G 2P5, Canada.

Abstract

Background and purpose: The study aimed to develop a localized topical anti-inflammatory treatment using a Thai medicinal herbal remedy called "Sahasthara," known for its anti-inflammatory properties, to create a film-forming spray (FFS).
Experimental approach: This research evaluated and developed an FFS formulated with Sahasthara ethanolic extract (SHTe). Subsequently, the optimized formulation was investigated for *in vitro* anti-inflammatory activity, cell culture toxicity assessment, pharmacological effects, and stability studies.
Findings/Results: An optimized formulation (F12) was identified, consisting of 1% w/w SHTe and PVP K90, glycerol, PEG 400, sesame oil, a eutectic blend, and ethanol. This clear, smooth surface, yellowish film releases 42.37%, 38.67%, and 68.93% at 8 h, corresponding to a flux of 20.94, 1.92, and 26.32 µg/cm^2/h of piperine, plumbagin, and β-asarone, respectively. F12 was determined to have a viscosity, drying time, and spray angle of 20 cps, 4.57 min, and 66.0 degrees. *In-vitro* anti-inflammatory activity demonstrated nitric oxide (NO) inhibition with an IC_{50} of 9.18 µg/mL. No apparent toxicity was observed in a skin cell line. This formulation was developed to be physically stable after undergoing freeze-thaw cycles. Although thermodynamic stability studies under accelerated conditions revealed a minor decrease in piperine and β-asarone within the film, the results indicate no statistically significant changes in its anti-inflammatory activity.
Conclusion and implications: SHTe FFS offers optimal spray ability, a high *in-vitro* drug release profile, potent inhibition of anti-inflammatory markers, and stability under accelerated conditions. These findings suggest that SHTe FFS can serve as an innovative topical anti-inflammatory treatment.

Keywords: Anti-inflammatory; Drug release; Film-forming spray; Sahasthara; Stability study; Topical drug.

INTRODUCTION

Inflammatory diseases are a major public health concern, and their prevalence is increasing globally. The inflammation process is often a protective reaction that takes place as a biological response to normal physiological and immune responses that occur at a local site, which can be triggered by pathogens, damaged cells)1), or irritants that consist of both vascular and cellular responses (2). The characteristics of inflammation are redness, swelling, heat, pain, and loss of tissue function (3). When activated, inflammatory cells (neutrophils, eosinophils, mononuclear phagocytes, and macrophages) produce an excess of inflammatory mediators and pro-inflammatory cytokines such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), interleukin (IL)-1ß, IL-6, and tumor necrosis factor (TNF) (1).

*Corresponding author: A. Itharat
Tel: +66-29269749, Fax: +66-29269705
Email: iarunporn@yahoo.com

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These substances have been described as mediators of inflammation and are used as important biomarkers (4), and therefore are useful targets in the development of anti-inflammatory dosage forms. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most utilized medications for treating inflammation and pain (5). Nevertheless, the continuous oral administration of NSAIDs can cause unwanted adverse effects such as gastrointestinal ulcers, bleeding, and renal complications due to non-selective inhibition of both cyclooxygenase (COX)-1 and COX-2. Localized treatment for anti-inflammatory delivery can also decrease adverse drug events by reducing systemic exposure.

The skin is an important route for dermal or transdermal delivery of pharmaceutically active substances (6). The outermost layer of the skin, the stratum corneum, is composed of dead, keratinized epidermal cells with a thickness of 10-20 µm and serves as a barrier for drug permeation (7). One topical drug delivery system commonly utilized over the past few years is film-forming formulations (8). Topical film-forming sprays (FFS) are a semi-solid dosage form that adheres to the skin surface, are less greasy than ointments, are almost invisible, and increase patient compliance by reducing application frequency (9). The film consists of piperine, plumbagin, and β-asarone, the main active compounds associated with anti-inflammatory properties, film-forming polymers, plasticizers, solvents/solubilizers, and other additives. After spraying onto the skin, the solvent evaporates, leaving a thin, transparent polymeric film (10). The film allows the active ingredients to diffuse across and into the skin (7), and this has many advantages over other delivery systems such as patches or semisolid preparations like creams, ointments, and gels (6,11).

Isolated plant-based molecules have long served as a rich source of potential new drugs (5). Traditional remedies are considered a safe and natural option for treating various illnesses. Thai Traditional Medicine (TTM) is an ancient medical system that treats people concerned with their well-being and seeks to restore a health imbalance.

Sahasthara, a pungent-tasting remedy from the Thai National List of Herbal Medicinal (NLHM) products, comprises 21 herbs and has long been used as a muscle painkiller and anti-inflammatory remedy for treating musculoskeletal disorders (12). Sahasthara ethanolic extract (SHTe) has been shown in earlier studies to have potent anti-inflammatory properties by competitively inhibiting NO production in lipopolysaccharide (LPS)-induced murine macrophages (RAW246.7) and reducing COX-2 protein levels with IC_{50} values of 2.81 µg/mL and 16.97 µg/mL, respectively (13). No apparent liver or kidney toxicity was found in male rats by intragastric feeding with 1,000 mg/kg/day of Sahasthara for 28 days (14). In addition, some clinical studies have reported utilizing SHT capsules to alleviate symptoms of muscle pain (15) and osteoarthritis (16). Moreover, topical 0.5 and 1% SHT demonstrated safety in 12 healthy volunteers using a cloth patch test (17). However, the SHT remedy as a topical FFS has not yet been investigated.

The current study aimed to develop a topical FFS containing SHTe as an anti-inflammatory treatment. The developed formulation was evaluated for its physicochemical properties, cell toxicity, and pharmacological effects.

MATERIALS AND METHODS

Plant material

The plant parts used in SHT are listed in Table 1. An herbalist authenticated all plant specimens, and voucher samples were preserved as verified crude specimens of Thai medicinal plants at the Thai Traditional Medicine Herbarium (TTM), under the Department of Thai Traditional and Alternative Medicine in Bangkok, Thailand.

Chemicals

Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) 400 were acquired from Srichand United Dispensary (BKK, Thailand). Glycerol, menthol, and camphor were provided by the PC Drug Center (BKK, Thailand). Sesame oil was purchased from Talaypu Natural Products Co., Ltd. (PNB, Thailand). Eudragit® RLPO and Ethyl cellulose (EC). Hydroxypropyl cellulose (HPC) was received as a gift sample from Evonik GmbH (Darmstadt, Germany).

Table 1. Botanical data of plants in Sahasthara remedy and the voucher specimens.

Taxonomic name (Family)	Part used	Place for collection	Voucher specimen (TTM No.)	Proportion in remedy (%)
Acorus calamus L. **(Acoraceae)**	Rhizome	Ratchaburi, Thailand	1000424	8.8
Anethum graveolens **L. (Apiaceae)**	Fruit	India	1000431	1.0
Atractylodes lancea **(Thunb.) DC. (Asteraceae)**	Rhizome	China	1000438	0.5
Baliospermum solanifolium **(Burm.) Suresh (Euphorbiaceae)**	Stem	Pathum Thani, Thailand	1000425	8.0
Cuminum cyminum **L. (Apiaceae)**	Fruit	India	1000434	0.8
Camphor (C10H16O)	Camphora syntheticum	Bangkok, Thailand	1000427	1.4
Ferula assa-foetida **L. (Apiaceae)**	Resin	China	1000432	1.0
Ipomoea obscura **(L.) Ker Gawl. (Convolvulaceae)**	Root	Samut Prakan, Thailand	1000435	0.8
Kleinhovia hospita **L. (Malvaceae)**	Stem	Chanthaburi, Thailand	1000426	4.8
Lepidium sativum **L. (Brassicaceae)**	Seed	India	1000430	1.1
Myristica fragrans **Houtt. (Myristicaceae)**	Arillode	China	1000428	1.3
Myristica fragrans **Houtt. (Myristicaceae)**	Seed	China	1000429	1.2
Nigella sativa **L. (Ranunculaceae)**	Seed	India	1000436	0.7
Neopicrorhiza scrophulariiflora **(Pennell) D. Y. Hong (Plantaginaceae)**	Rhizome	China	1000439	0.4
Pimpinella anisum **L. (Apiaceae)**	Fruit	India	1000433	0.9
Pistacia chinensis **subsp. integerrima (J. L. Stewart ex Brandis) Rech. f. (Anacardiaceae)**	Root	China	1000437	0.6
Plumbago indica **L. (Plumbaginaceae)**	Root	Kanchanaburi, Thailand	1000421	22.4
Piper nigrum **L. (Piperaceae)**	Fruit	Chanthaburi, Thailand	1000420	24
Piper retrofractum **Vahl (Piperaceae)**	Fruit-spike	Chanthaburi, Thailand	1000423	9.6
Terminalia chebula **Retz. (Combretaceae)**	Fruit	Prachin Buri, Thailand	1000422	10.4
Terminalia chebula **Retz. Var. chebula (Combretaceae)**	Gall	China	1000440	0.3

Fetal bovine serum (FBS) and penicillin-streptomycin were procured from Gibco® BRL Life Technologies (NY, USA). LPS (serotype: *Escherichia coli* O55:B5) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich® Inc. (MO, USA). All other chemicals and reagents used were of analytical grade and were commercially sourced.

Preparation of SHTe

Each part of the plant material was cleaned, crushed into a moderately coarse powder, weighed according to the prescription, and mixed. The extract was obtained through maceration with 95% ethanol for 3 days, with occasional shaking. Subsequently, the liquid was strained, and the remaining liquid was reclaimed by further pressing. This process of re-extraction was repeated two more times. Following these extractions, the extract was filtered and then dried using a rotary evaporator at 45 °C until the solvent had completely evaporated (Rotavapor R-205, Buchi, Germany). The resulting product was stored in a freezer at -20 °C until further experimentation. The percentage yield of the extract was then calculated.

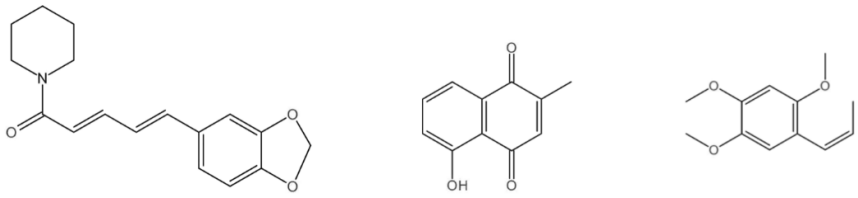

Fig. 1. The structure of the main chemical constituents found in Sahasthara ethanolic extract.

Table 2. HPLC gradient mobile phase for the analysis of chemical fingerprints in Sahasthara ethanolic extract.

Step	Time) min(	Acetonitrile)B(	0.1 %Phosphoric acid)A(
1	0	5	95
2	5	5	95
3	40	50	50
4	45	95	5
5	55	100	0
6	55.1	5	95
7	60	5	95

Analysis of the chemical fingerprint of SHT

A high-performance liquid chromatography (HPLC) method was used to determine the content of piperine, plumbagin, and β-asarone, the main compounds in SHT that were associated with anti-inflammatory properties (structures are shown in Fig. 1). The HPLC system (Agilent® 1200) was equipped with a solvent degasser (G1322A), a quaternary solvent pump (G1311A), an autosampler (G1329A), a column oven (G1316A), and a photodiode array detector (G1315D). Chromatographic separation was performed at 25 ± 1 °C using a reverse-phase C18 column (Phenomenex, 5 um, 4.6 × 250 mm, USA). Ten microliters were injected into the HPLC system, and the samples were eluted using a gradient mobile phase consisting of 0.1% phosphoric acid (A) and acetonitrile (B) with various ratios as shown in Table 2, using two wavelengths of 256 and 310 nm at a flow rate of 1.0 mL/min.

Preparation of SHTe film-forming spray

In this study, all formulations (FA-FF) containing 1% SHTe were prepared using various polymers and other ingredients as outlined in Table 3. SHTe was dissolved in 30 mL of absolute ethanol while stirring for 10-12 h until a homogeneous solution was achieved. Simultaneously, the film-forming agent, plasticizer, penetration enhancer, and residual solvent were mixed dropwise under constant slow stirring. Subsequently, the SHTe solution was blended with the film solution, and the mixture was continuously stirred until complete dissolution, resulting in a 1.0% w/w SHTe concentration. After evaluating the film characteristics, sesame oil and a eutectic blend (menthol and camphor) were added to the six new formulations (FG-FL), as indicated in Table 4, to enhance SHTe absorption into the skin. This solution was then promptly transferred to a spray bottle to avoid solvent evaporation. The formulation exhibiting the ideal properties was selected for optimization using a factorial design through the Design of Experiment (DoE) program. Factors were assessed at various concentrations ranging from their minimum to maximum values. Finally, at least one experimental trial was conducted on the optimized formulation.

Evaluation of mechanical film properties

To evaluate the mechanical properties, various formulations of FFS were cast in a Teflon mold (60 × 80 mm², 12 mL) at 45 °C in a hot air oven for 48-72 h. Once completely dried, the films were carefully peeled off, and the thickness of the formed films was measured using a vernier caliper at three positions along their length (18). Moreover, the films were cut into appropriate sizes for evaluating tensile strength and percent elongation using a TA-XTPLUS texture analyzer (Stable Micro System, Cardiff, UK).

Table 3. Composition of the formulation; compound content in percentage)w/w(; and film characteristics.

Formulation	Eudragit RLPO	HPC	PVP-K30	PVP-K90	EC	Stearyl alcohol	PEG400	Glycerol	EtOH 65%	EtOH 95%	EtOH and acetone	Film characteristics
FA	3.25	3.25					1		q.s.			The complete film with no cracks and a slight flexibility
FB	10				5		0.45				q.s.	The complete film with no cracks or flexing
FC			5			7.5	5			q.s.		Completely missing film)some stuck in the bottle(.
FD			4			2.5	2.5			q.s.		Missing film sections and sporadic flaking
FE				5			2	1		q.s.		The complete film with no cracks and a slight flexibility
FF				5		5	5			q.s.		Missing film sections and sporadic flaking

HPC, hydroxypropyl cellulose; PVP, polyvinylpyrrolidone; EC, ethylcellulose; PEG, polyethylene glycol; q.s., quantum satis or "a sufficient quantity".

Table 4. The composition of the formulation, the compound content in percentage)w/w(, and the formulation properties. Data are presented as mean ± SD; n =3.

Formulation	Ingredients %)w/w(											Formulation properties			
	Eudragit RLPO	HPC	PVP-K90	EC	PEG 400	Glycerol	Eutectic blend 1 :1	Sesame oil	EtOH 65%	EtOH 95%	EtOH and Acetone	Thickness^{*a})mm(	Tensile strength^{*A})N/mm2(	Elongation at break^{*A} (%)	Viscosity^{*K})mPa.s(cP
FG	3.25	3.25			0.35		0.1	1	q.s.			0.19 ± 0.02^{a}	153.77 ± 5.26^{a}	26.28 ± 3.25^{a}	82.22 ± 0.14^{a}
FH	3.25	3.25			0.5		0.1	1	q.s.			0.23 ± 0.02^{a}	163.67 ± 15.03^{a}	44.56 ± 2.77^{b}	85.82 ± 0.42^{a}
FI	2.5	2.5			0.2		0.1	1	q.s.			0.19 ± 0.01^{a}	162.51 ± 1.48^{a}	24.68 ± 2.24^{a}	43.95 ± 0.50^{b}
FJ	5			2.5	0.45		0.1	1			q.s.	0.36 ± 0.02b	78.62 ± 4.90^{b}	69.28 ± 17.81c	12.24 ± 0.12^{c}
FK			5		0.5	1	0.1	1		q.s.		0.19 ± 0.02^{a}	2.14 ± 0.08^{c}	186.99 ± 0.92^{d}	21.31 ± 0.66^{c}
FL			2.5		0.5	1	0.1	1		q.s.		0.14 ± 0.02^{a}	7.06 ± 0.37^{c}	316.66 ± 7.64^{e}	11.04 ± 0.12^{c}

^{a,b,c,d,e} $P < 0.05$ indicate pairwise significant difference; ^{*A} One-way ANOVA, ^{*K}Kruskal-Wallis 1-way ANOVA; HPC, hydroxypropyl cellulose; PVP, polyvinylpyrrolidone; EC, ethylcellulose; PEG, polyethylene glycol; q.s., quantum satis or "a sufficient quantity".

Tensile strength measures a material's ability to withstand stretching and tearing under load, while percentage elongation indicates its ability to stretch without breaking (8). Following the method by Vij *et al.*, films with dimensions of 15 × 60 mm were attached to a support, which was subsequently held between two grips with an initial distance of 40 mm. The extension rate was set at 2 mm/s. During the measurement, weights were added, and the strength and elongation were measured when the films broke, using equations (1) and (2).

$$\text{Tensile strength} = \frac{\text{Tensile load at break}}{\text{Cross sectional area}} \quad (1)$$

$$\text{Elongation (\%)} = \frac{\text{Maximum length recorded at break-original length}}{\text{Original length}} \times 100 \quad (2)$$

Spray pattern and angle

Spray patterns were characterized by adding a few drops of methylene blue to all formulations and mixing them uniformly. The solution was then sprayed horizontally onto white paper placed 15 cm from the nozzle to visualize the spray pattern after application. The spray angle was calculated using the arctan ($\tan^{-1}$) of the ratio of the distance between the paper and the nozzle to the average radius of the circle in triplicate directions, as depicted in equation (3) (19-21).

$$\text{Spray angle } (\theta) = \tan^{-1}\left(\frac{\text{distance}}{\text{radius}}\right) \quad (3)$$

In-vitro drug release study

Franz-type diffusion cells with a diffusion area of 1.77 cm² were used to evaluate the *in-vitro* release profiles from the FFS. Dry film samples were cut and applied to a dialysis membrane (CelluSep® T4, USA). The receptor medium was a pH 7.4 phosphate buffer solution containing 10% v/v ethanol (12 mL). The temperature of the receptor medium was maintained at 32 ± 0.5 °C and stirred at 600 rpm. One mL of the receptor medium was withdrawn at specified intervals (0.5, 1, 2, 4, 6, and 8 h) and replaced with an equal volume of fresh buffer. The withdrawn medium was then filtered through a syringe filter with a 0.45 µm pore size. The experiment was conducted in triplicate and subsequently analyzed for compound content using HPLC, following the method described by Kakatum *et al.* (22), which was previously developed and validated according to ICH guidelines. The cumulative amount of active compounds was plotted against time.

Transmittance

The percentage of transmittance for the optimized formulation was determined by diluting 1 mL of each formulation (1:99) with deionized water and measuring it using a spectrophotometer at a wavelength of 550 nm, with deionized water used as a control.

Viscosity measurements

The viscosity of the solution was measured using a Brookfield viscometer (LV) at 25 ± 1 °C with an SC4-31 spindle rotated at 250 rpm. Each measurement was taken throughout 10 points, with a time interval of 1 min. An average of three readings was then reported (18).

Evaporation time and stickiness

For the evaporation time test, 100 µL of FFS was deposited onto aluminum foil measuring 50 × 20 mm^2 and evaporated completely using a moisture analyzer set at 50 °C. After a specific time interval, a glass slide was gently placed on the film without applying pressure, and the time required for the spray film to dry was recorded. The stickiness of the films was assessed by lightly pressing cotton fibers onto the dry film under low pressure, resulting in ratings such as '+++' for a dense layer of fibers on the film, '++' for a thin layer of fibers, or '+' indicating temporary or no adherence of fibers (23).

Surface morphology

The surface morphology of the SHTe FFS was examined using field emission scanning electron microscopy (FE-SEM; JEOL JSM7800F, Japan). The dry film was affixed to the studs using carbon tape and subsequently gold coated (QUORUM Q150R ES, UK). The prepared studs were directly analyzed at an increasing voltage of 5 kV.

Thermodynamic stability studies

Freeze-thaw

The SHTe FFS was stored at 0 °C for 48 h and at 25 °C for 4 h per cycle. The test was conducted over five cycles. Measurements of the three chemical constituents (piperine, plumbagin, and β-asarone) were determined in triplicate using the HPLC method and were also assessed visually for appearance and phase separation (24).

Accelerated stability study

A stability study under accelerated conditions for the selected SHTe FFS was conducted for 6 months at 40 ± 2 °C and 75 ± 5% relative humidity, following the guidelines of the Thai FDA (25). Changes in visual appearance, content of marker compounds, and anti-inflammatory activity were assessed at various storage times (0, 15, 30, 60, 90, 120, 150, and 180 days).

Anti-inflammatory efficacy studies

Inhibition of NO production assay in RAW 264.7 cells

Mouse macrophage-like cells (RAW 264.7; ATCC® TIB-71TM) were cultured in Roswell Park Memorial Institute (RPMI) medium with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin (10 units/mL) at 37 °C in a 5% CO_2 incubator. Cells were seeded in a 96-well sterile plate (1×10^6 cells/well) with 100 µL of complete medium and incubated for 24 h. Subsequently, the medium was replaced with 100 µL of complete medium containing 5 ng/mL of LPS, and then 100 µL of samples were added to achieve final concentrations ranging from 0.1 to 30 µg/mL with 0.2% DMSO. The cells were further incubated for an additional 24 h. NO production was determined by measuring the accumulation of nitrite, which reacted with the Griess reagent. After the procedure, 100 µL of supernatant was transferred to another 96-well plate, followed by the addition of 100 µL of Griess reagent. Absorbance was measured using a microplate reader at a wavelength of 570 nm (26). The inhibition (%) was calculated using equation (4).

$$\text{Inhibition (\%)} = \frac{(C\text{-}S)}{C} \times 100 \quad (4)$$

where C and S stand for the optical density of the control and sample, respectively.

Cell viability by MTT assay

A cell viability test was conducted to verify the decrease in NO production after exposure to the extract. The MTT colorimetric method was employed to assess the cytotoxicity of the extracts on RAW 264.7 cells at various concentrations. Following a 24-h exposure to the extract, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well, and the cells were incubated for an additional 2 h before removing the supernatant. The purple formazan produced by the cells was dissolved in 100 µL of isopropanol containing 0.04 M HCl, and the absorbance was measured at 570 nm. Cell viability was calculated using equation (5). If the cell viability exceeded 70%, it was considered indicative of survival.

$$\text{Cell viability (\%)} = (100 - \frac{C - S}{C}) \times 100 \quad (5)$$

where C and S stand for the optical density of the control and

Skin cell viability test in HaCaT cells

This assay was employed to assess the toxicity of FFS on human skin cells *in vitro*. The immortalized human keratinocyte cell line (HaCaT) was cultured in Dulbecco's Modified Eagle Medium (DMEM), and the cell culture protocol was as above. The cells were seeded in a 96-well sterile plate at 5 × 103 cells/well density and incubated for 24 h. Afterward, the complete medium (100 µL/well) was replaced, and then 100 µL of samples were dissolved in the medium to achieve final concentrations ranging from 0.1-30 µg/mL, along with 0.2% DMSO. The cells were then incubated for another 24 h. Subsequently, 10 µL of MTT reagent was added into each well and allowed to incubate for 2 h before measuring cell viability at a wavelength of 570 nm.

Statistical analysis

The data are presented as the mean ± SD or SEM for continuous variables using Microsoft Excel® software. The IC_{50} was calculated using GraphPad Prism software (GraphPad Software, Inc.). All statistical calculations were conducted with the Statistical Package for Social Sciences (SPSS) software program, with statistical significance defined as $P < 0.05$.

RESULTS

Preparation of SHTe

The SHTe solution exhibited a clear, slightly dark brown color, with a yield of 10.28% w/w based on the weight of dry SHT. HPLC chromatograms of SHTe and the standard substances plumbagin, piperine, and β-asarone are depicted in Fig. 2A and B. The chromatogram peaks for SHTe and the standards appeared at the same retention time, confirmed by the increased area under the curve, as shown in Fig. 2C when spiked with SHTe and all the standards in the same injection. The content of plumbagin, piperine, and β-asarone, as determined by the HPLC technique, was 5.62, 118.06, and 36.33 mg/g, respectively. Linear regression equations for the determination of the contents of active compounds are presented as equations (6), (7), and (8).

Concentration plumbagin = (Areaplumbagin + 46.871) / 36.184, $R^2 = 0.9993$ (6)

Concentrationpiperine = (Areapiperine + 304.900) / 19.080, $R^2 = 0.9999$ (7)

Concentrationβ-asarone = (Areaβ-asarone + 574.140) / 33.931, $R^2 = 0.9990$ (8)

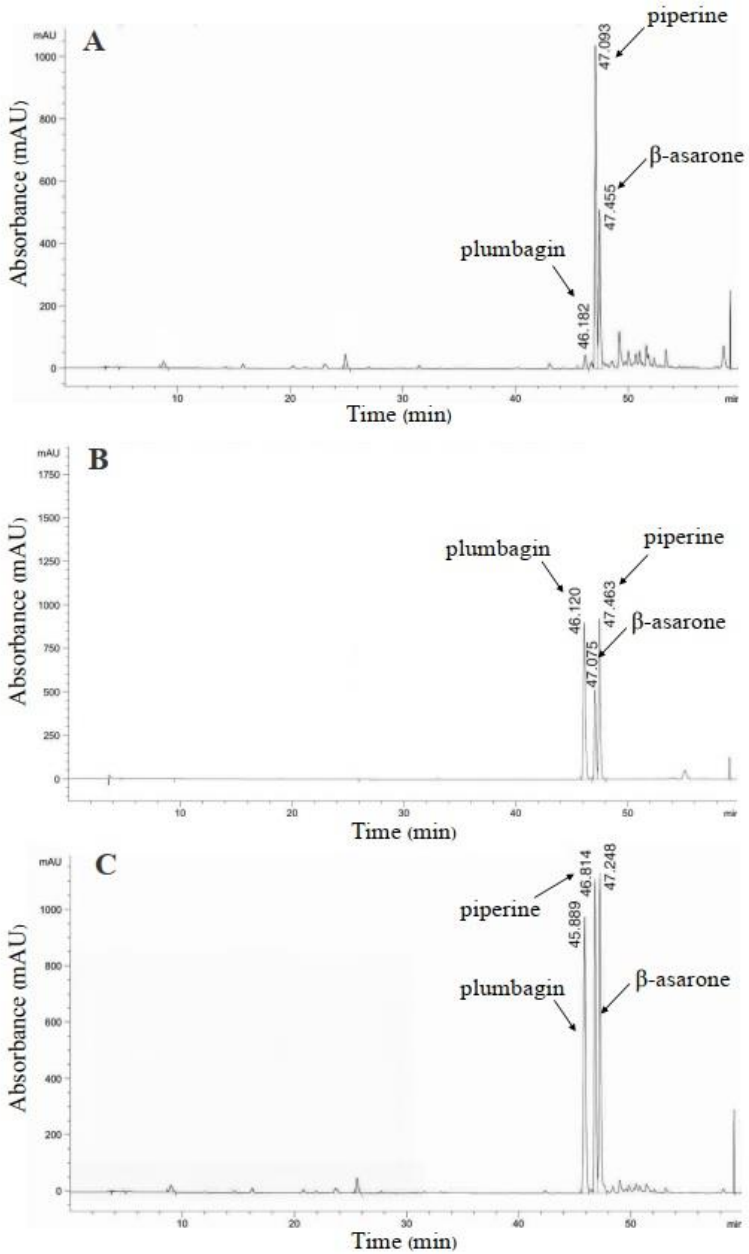

Fig. 2. Chromatograms of (A) plumbagin, piperine, and β-asarone in SHTe; (B) standard plumbagin, piperine, and β-asarone; and (C) SHTe and all standards in the same injection. SHTe, Sahasthara ethanolic extract.

Preparation of SHTe film-forming spray and film characteristics

The organic solvents used in topical drug delivery systems for SHTe, which is insoluble in water, include ethanol and acetone. These solvents not only remove stratum corneum lipids (27) but also render the solution transparent, clear, and homogeneous. The study revealed that SHTe was completely soluble in both solvents, resulting in all formulations having a yellow color. Films produced from FA, FB, and FE were complete, non-flaky, with no cracks, and slightly flexible (FA and FE) or not flexible (FB). However, all the films exhibited a slight stickiness when touched. FC, on the other hand, not only had many white particles that remained undissolved at the bottom of the container but also experienced film rupture in almost all parts, tending to form gnarled spheres and laminates on the dish after drying. FD and FE formed films that were partially cracked and had sporadic flaking due to the brittleness of the film. Based on the data above, FC, FD, and FF were eliminated from the investigation. FA, FB, and FE were selected for further study by reducing the polymer concentrations due to the stickiness they exhibited. Additionally, sesame oil was added due to its ability to enhance the absorption of water-insoluble drugs into the skin, promote transdermal permeation, and possess non-toxic properties for skin cells. To further enhance *ex-vivo* drug diffusion, menthol and camphor (eutectic blend) were also included in all formulations, as they have been shown to improve drug release through rat skin and act as dermal penetration enhancers (28). As a result, new formulations were obtained as presented in Table 4.

Preparation of SHTe film spray and properties

Six formulations were developed and tested for mechanical properties, viscosity, and *in-vitro* release using Franz diffusion cells (mean ± SD, n = 3). The results showed that the thickness of all formulations ranged from 0.19 to 0.36 mm. Ammar *et al.* found that a successful formulation for prolonged drug delivery requires a highly flexible film to accommodate skin movement (29); therefore, FL exhibited the most appropriate tensile strength and elongation properties. The viscosity of all liquid formulations ranged from 11.04 to 85.82 cP. The results from the *in-vitro* diffusion study for FG to FL over 8 h are illustrated in Fig. 3.

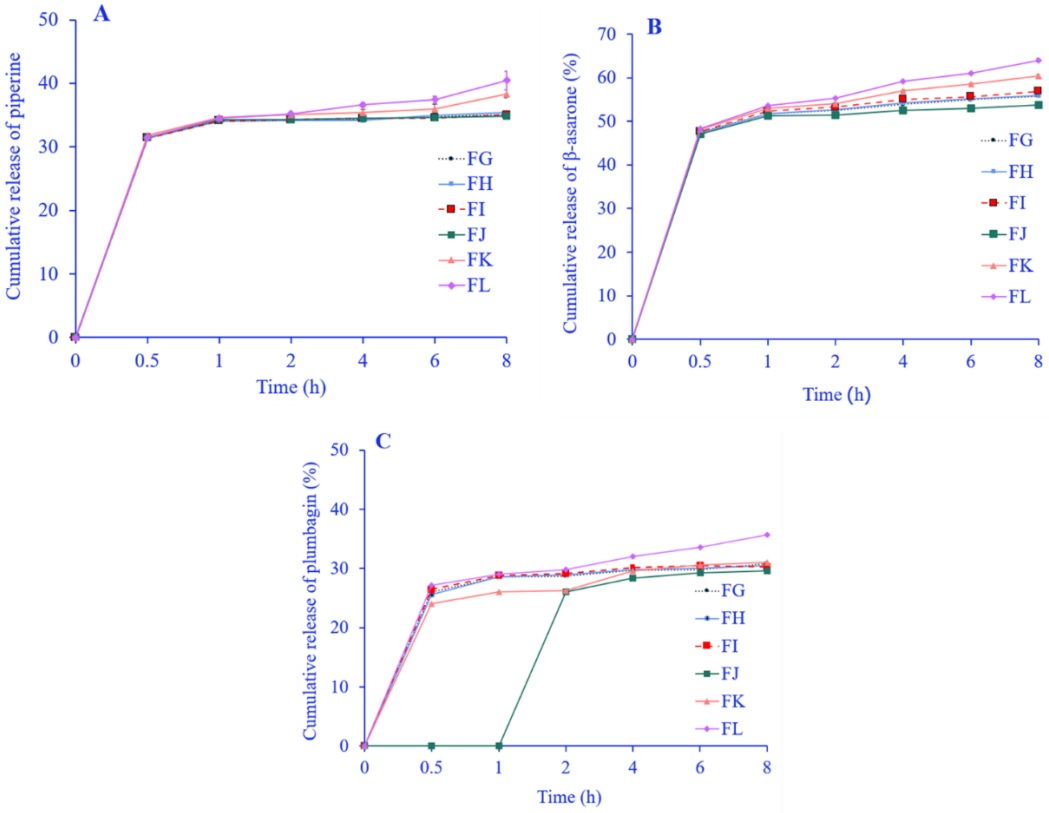

Fig. 3. Comparison of the percent cumulative drug release profiles of FG to FL versus time (8 h) for (A) piperine, (B) plumbagin, and (C) β-asarone.

The release profiles indicated that none of the formulations completely released the marker molecules. FL exhibited the highest release rate among all the chemical components, suggesting that this formulation might have the highest anti-inflammatory activity compared to the others, followed by FK and FH, respectively.

Considering all parameters, it can be concluded that FL performed *in vitro* as the best FFS with the highest drug release. Selected formulations (FL) were then investigated for mixture composition to achieve the best pharmaceutically acceptable topical film spray formula.

Preparation of the optimized formulations by Mixture Design

DoE is presented in Table 5. Spray ability was employed to ascertain the suitable formulation ratio. The experiment was designed using the optimal (low-high) values for three variables (PVPK 90, PEG 400, and glycerol) that were correlated with the concentration of the film-forming agent while keeping the values of other ingredients fixed (0.1% eutectic blend, 1% sesame oil, and 1% SHTe, all at the same concentrations for all formulations). The solvent used was 95% ethanol.

Spray angle

The average spray angle from F1 to F11 ranged between 53.97° and 65.32°. Previous research suggests an acceptable spray angle should be less than 85° for ease of actuation of the drug solution and coverage of a surface area (9,19). Therefore, a target angle of 66° was selected. The optimized formulation, F12, produced a homogeneous film with minimal outward stickiness and a slight sticky sensation at the application site.

Spray pattern

The spray pattern for the formulation batches exhibited a satisfactory pattern regarding uniformity and spherical spots. However, spray patterns rely on the nozzle and the local vendor's spray pumps did not generate a consistent spray pattern, resulting in scattered droplets with drops of various sizes (Fig. 4). F12 was shown to have an average spray weight of 0.11 ± 0.01 g.

Table 5. Composition of Mixture Design (Design of Experiment; DoE) to optimize the best formulation.

Formulation	Excipients			Spray angle
	Glycerol	PVP K90	PEG 400	
F1	1.00	1.50	1.50	60.32
F2	1.60	1.75	0.65	61.14
F3	0.85	2.625	0.525	56.96
F4	0.50	3.25	0.25	53.97
F5	0.50	2.00	1.50	61.05
F6	1.20	2.00	0.80	60.78
F7	2.00	1.50	0.50	65.32
F8	2.00	1.75	0.25	59.97
F9	0.85	2.00	1.15	64.01
F10	1.10	1.75	1.15	60.01
F11	1.60	1.875	0.525	58.78
F12	1.50	1.50	1.00	66.00

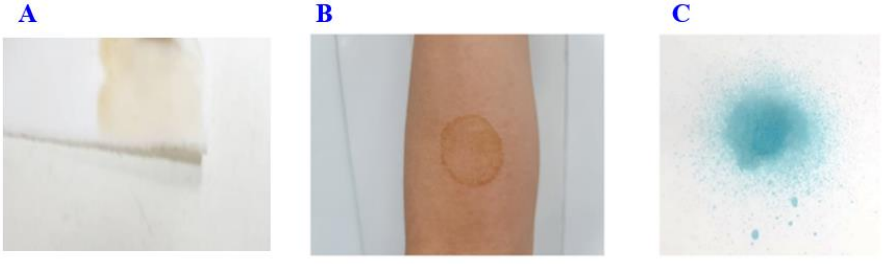

Fig. 4. The appearance of the optimized film-forming spray after drying on (A) a Teflon sheet, (B) forehand skin, and (C) a spray pattern on paper.

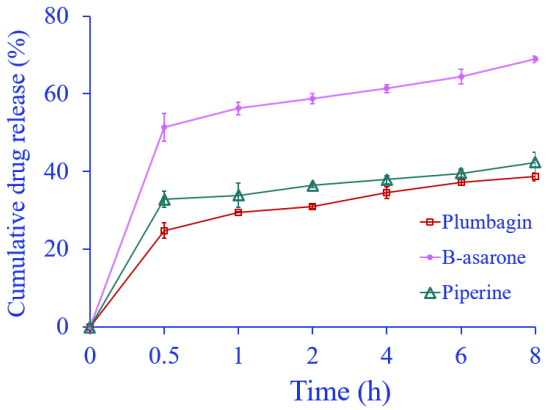

Fig. 5. Cumulative release profiles of active compounds from the optimized formulation (F12).

In-vitro drug release study

The release kinetics of the optimized formulation across the cellulose membrane is shown in Fig. 5. The percentage of drug released up to 8 h was 42.37%, 38.67%, and 68.93%, corresponding to a flux of 20.94, 1.92, and 26.32 µg/cm^2/h, for piperine, plumbagin, and β-asarone, respectively.

Chemical property testing

The results of content uniformity indicated that the average drug content per cm^2 of the dry-prepared formulation was 15.85 ± 0.10, 1.49 ± 0.01, and 5.90 ± 0.01 mg/cm^2 for piperine, plumbagin, and β-asarone, respectively.

Transmittance

The percent transmittance of F12, when compared to deionized water, was 99.06 ± 0.05%, indicating that the formula is nearly as transparent as water.

Viscosity measurement

The viscosity of F12, a term representing a substance or chemical (consider capitalizing or italicizing it for clarity), was 20 cps, providing a suitable balance between spray capacity and the required viscosity to ensure the spray adheres to the skin, creating a comprehensive and smooth film that does not drip until it dries. Similarly, Ranade *et al.* and Mori *et al*. reported that the optimal viscosity for effective spray ability was less than 50 mPa.s. and 80 cps, respectively (21,9).

Evaporation time and the stickiness of the film

This test investigated the effectiveness of the solvents in evaporating and forming a film. The moisture analyzer measured the evaporation time of F12 at 50 °C, resulting in a time of 5.23 ± 0.03 min.

Surface morphology

FE-SEM was used to examine the surface morphology of FFS. In Fig. 6 magnifications at ×100, ×1000, and ×5000 are shown. At ×5000 magnification, a wave-like appearance is visible, and natural oil spheres are scattered across the film's surface.

Determination of in-vitro anti-inflammatory activity

F12 strongly inhibited NO production in a concentration-dependent manner, as shown in Fig. 7A and B, and the inhibition reached 85.42% at a concentration of 30 µg/mL. The results showed that F12 greatly inhibited NO production with an IC_{50} of 9.18 ug/mL, which was more effective than the NSAID diclofenac, but less effective than prednisolone. Interestingly, a previous report indicated that SHTe exhibits a higher NO production inhibitory effect than the NSAID indomethacin, with an IC_{50} of 56.8 µM or 20.3 µg/mL (13).

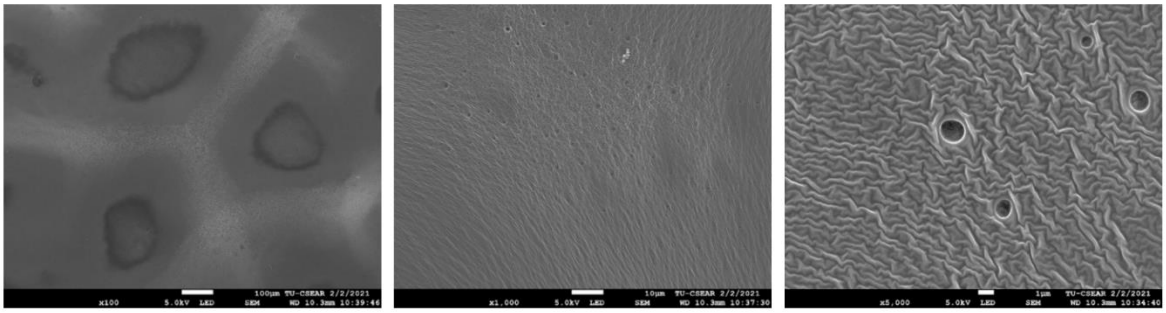

Fig. 6. FE-SEM images of the optimized film-forming spray at a concentration of 1% Sahasthara ethanolic extract (5 kV with a magnification of 100, 1,000, and 5,000).

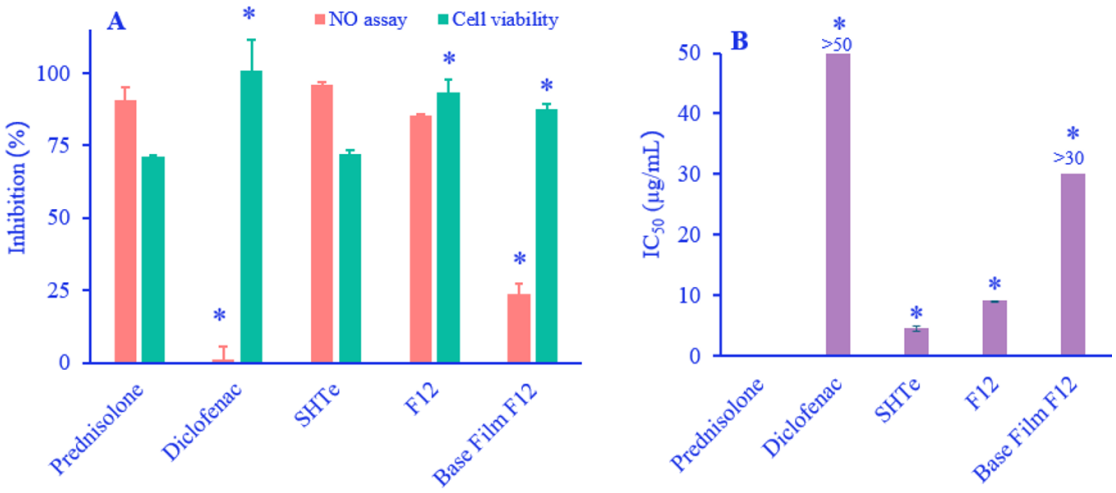

Fig. 7. (A) The percentage of LPS-induced NO production inhibition (%) in RAW264.7 cells and cell viability at the concentration of 30 µg/mL of Sahasthara ethanolic extract, F12, and base film F12; (B) the IC_{50} of each extract on LPS-induced NO production in RAW264.7 cells compared to the standard positive control (prednisolone and diclofenac). Data are expressed as mean ± SEM, n = 3. *P < 0.05 indicates significant differences compared to the positive control, prednisolone. LPS, Lipopolysaccharide; NO, nitric oxide.

Table 6. The viability of HaCat cells at various concentrations of F12 and SHTe. Data are presented as mean ± SEM, n = 3).

Sample	0. 1 µg/mL	1 µg/mL	10 µg/mL	30 µg/mL
F12	96.03 ± 0.59	95.94 ± 1.07	90.81 ± 1.58	84.69 ± 0.02
SHTe	99.36 ± 0.23	95.46 ± 0.31	88.02 ± 0.50	78.23 ± 0.59

SHTe, Sahasthara ethanolic extract.

Cell viability after treatment with all samples was determined using the MTT assay as described above. These results demonstrated that none of the extracts revealed any cytotoxic effects at the test concentration, with more than 70% cell viability. These findings suggest that the inhibition of LPS-induced NO synthesis in the treated cells was due to NO inhibition by these extracts rather than cell death (30).

Skin cell toxicity

The results of the *in-vitro* keratinocyte toxicity test on HaCat cells are presented in Table 6. It was concluded that at 30 µg/mL, both F12 and SHTe exhibited more than 70% cell viability and did not exhibit toxicity to skin cells at the test concentrations.

Thermodynamic stability studies

Freeze-thaw

After five freeze-thaw cycles, the physical appearance of FFS in terms of transparency and phase separation remained unchanged. The pure compound content did not change significantly after all cycles. Therefore, the formulations were considered physically stable.

Stability studies under accelerated conditions

The six-month stability test was carried out by storing FFS in a climate-controlled chamber. The results of this test are presented in Table 7. During the test, there was an insignificant change in percent transmittance. For active compounds, piperine and β-asarone did not show any statistical change. Only plumbagin decreased by over 50% and changed at day 180 (compared with day 0). For anti-inflammatory activity, F12 revealed a negligible decrease, less than 30 µg/mL, which is acceptable as potent biological activity (31).

Evaluation parameters for formulation optimization.

Various test parameters were evaluated for the final formulation quality Table 8. The quantity delivered in each activation, the amount of SHTe delivered, appearance, color, spray angle, transmittance (%), viscosity, evaporation time, stickiness of the film as well as inflammatory activity were all optimized.

Table 7. Changes in transmittance, active compounds, and anti-inflammatory activity of the optimized formulation after accelerated storage (0, 15, 30, 60, 90, 120, 150, and 180 days). Data are presented as mean ± SEM, n = 3.

Day	Transmittance (%)	Active compounds			Anti-inflammatory (nitric oxide assay)
		Piperine (mg/g) (% remaining)	β-asarone (mg/g) (% remaining)	Plumbagin (mg/g) (% remaining)	Film12 IC_{50} (µg/mL)
0	99.06 ± 0.05	69.63 ± 0.30	20.53 ± 0.38	5.24 ± 0.04	9.18 ± 0.14
15	98.90 ± 0.07	68.43 ± 1.05 (98.28)	20.27 ± 0.24 (98.73)	3.99 ± 0.31 (76.15)	11.83 ± 0.96
30	98.86 ± 0.04	66.15 ± 0.44 (95.00)	19.57 ± 0.12 (95.32)	3.74 ± 0.20 (71.37)	11.58 ± 0.05
60	98.87 ± 0.01	65.05 ± 0.26 (93.42)	19.22 ± 0.07 (93.62)	3.51 ± 0.15 (66.98)	10.41 ± 0.18
90	98.89 ± 0.04	66.53 ± 0.64 (95.55)	19.56 ± 0.11 (95.28)	3.22 ± 0.15 (61.45)	17.26 ± 0.52*
120	98.91 ± 0.05	66.10 ± 0.42 (94.93)	19.44 ± 0.14 (94.69)	2.94 ± 0.20 (56.11)	17.94 ± 0.56*
150	98.85 ± 0.03	65.74 ± 0.81 (94.41)	16.22 ± 0.17 (79.00)	2.21 ± 0.17 (42.18)	27.43 ± 0.64*
180	98.97 ± 0.01	65.11 ± 0.61 (93.51)	16.51 ± 1.09 (80.42)	1.59 ± 0.07 (30.34)*	27.03 ± 0.90*

*P < 0.05 indicates significant differences from day 0.

Table 8. Evaluation parameters related to the container for optimizing formulation. Data are presented as mean ± SD, n = 3.

Test parameters	Average results
The quantity delivered with each actuation	0.11 ± 0.00 mL
Amount of SHTe delivered upon each actuation	1.1 mg
Appearance of the film	Complete film with slight flexibility
Color	A clear, yellowish solution
Spray angle	66o
Transmittance (%)	99.06 ± 0.05
Viscosity	20 Cps
Evaporation time	4.57 min
Stickiness of the film	Little outward stickiness and a slight sticky sensation at the application site
Inflammatory activity (inhibitory of NO assay)	9.18 ± 0.14 µg/mL

DISCUSSION

Topical FFS systems that create a film on the skin are readily easy to pharmaceutically formulate and offer numerous benefits, including transparency, non-greasiness, resistance to being wiped off, greater dosage flexibility, and reduced skin irritation (20). For this research, Sahasthara remedy, derived from a traditional Thai scripture, was chosen. Traditional Thai medicine suggests that the numerous active phytochemical components in this remedy can effectively target multiple pathways, treating diseases through diverse mechanistic approaches. Piperine, obtained from *Piper nigrum* (black pepper) and *P. longum*, has previously been shown to inhibit iNOS and suppress the production of pro-inflammatory cytokine mRNA and protein by blocking the expression of IL-1, IL6, TNF-α, COX-2, and PGE2, as well as inhibiting the LPS-mediated activation of NF-κB and degradation of IκB-α (32-34). Similarly, plumbagin has been demonstrated to reduce the production of proinflammatory cytokines such as IL-1, IL-6, and TNF-α, as well as the expression of proinflammatory mediators such as iNOS and COX-2 (35,36). It was also determined that β-asarone suppressed the expression of IL-6, IL-1, iNOS, and COX-2 (37). Hence, SHTe appears to have the potential to prevent inflammation due to its anti-inflammatory activities affecting different signaling pathways.

Film-forming sprays of SHTe were prepared using various ingredients to achieve the desired film properties. (Table 8) Based on the obtained results, the most optimal formulation was selected based on its physical properties, and an investigation of its *in vitro* drug release was

performed. The optimized formulation containing SHTe, PVP, PEG 400, glycerol, a eutectic blend of menthol and camphor, sesame oil, and ethanol exhibited the most optimal penetration profile. PVP was considered a film-forming polymer due to its high hydrophilicity and non-ionic character (8). In addition, PVP has substantial moisture sorption properties (10). Because glycerin and PEG 400 are non-toxic plasticizers and can act as humectants, we used them in our study. PEG is widely used as a solubilizer or permeation enhancer, although it doesn't improve *in vitro* drug release (28). PEG 400 significantly influences the skin's barrier structure by lowering surface tension and conditioning the stratum corneum without causing skin irritation when formulated in low quantities (38), resulting in a longer evaporation time. Moreover, PVP and PEG 400 are employed as anti-nucleating agents and crystallization inhibitors for drugs, which are retained even after solvent evaporation. A eutectic blend of menthol and camphor was also included to improve drug uptake by altering the barrier function of the stratum corneum and increasing the permeability of drugs applied to the skin (19,39).

The concentrations of all selected polymers were optimized through statistical design to obtain the best formulation. F12, as shown in Table 8, produced homogeneous films with minimal outward stickiness and a slight sticky sensation at the application site. The film completely dried within 5 min in an open environment. The spray ability of the optimized formulation, set at 66.0 degrees, is greatly influenced by viscosity (40) and the ingredients of the FFS. As the spray angle increases, viscosity decreases due to a lower amount of polymer and plasticizer. Consequently, less polymer and plasticizer resulted in an increased spray angle. The optimized FFS, which contains medicinally active compounds, strongly inhibited NO production with an IC_{50} of 9.18 µg/mL and demonstrated beneficial therapeutic effects in managing inflammatory diseases, making it a viable alternative to some commercial products. However, the stability study of the optimized formulation under accelerated conditions revealed no change in transmittance or degradation of two active compounds, piperine and β-asarone. Conversely, transmittance decreased to only 30% remaining once for plumbagin compared with day 0. This might be due to the sublimation of this compound (41), which is unstable and sensitive to temperature. Thus, plumbagin should be stored at a low temperature or entrapped in liposomes to address this issue (42).

In summary, the development of SHTe FFS formulation holds great potential for topical delivery to treat inflammation. These findings are promising for developing this innovative topical drug delivery technology.

CONCLUSION

In this investigation, the SHTe FFS exhibited all the characteristics necessary for topical application. By selecting the most optimal combination of polymers and ingredients, the optimized formulation, containing 1% w/w SHT, PVPK90 as the film-forming agent, ethanol as the solvent, efficient plasticizers like PEG 400 and glycerol, sesame oil, and a eutectic mixture to enhance drug penetration, resulted in a film that was shiny, translucent, flexible, minimally sticky, soft to the touch, and consistently present with an appropriate spray angle. The anti-inflammatory activity was more effective than the positive control as determined by the inhibitory assay of NO. The stability investigation revealed negligible statistical variation. This study demonstrates that the optimized formulation is capable and effective for topical spray application, with promising potential for anti-inflammatory skin treatment.

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contributions

A. Itharat conceptualized and supervised the study. N. Intharit and C. Choochong conducted the experiments. N. Intharit wrote the manuscript with support from A. Itharat. R. Löbenberg and W. Pipatrattanaseree provided the technical method for HPLC and formal analysis. W. Ketjinda verified the analytical methods for drug release. R. Löbenberg and N.M. Davies provided scientific expertise and edited and revised the manuscript. All authors read and approved the final version of the article.

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