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Development of Dosage form of Ya Kae Fok Buam Mueai Khop, a Thai Traditional Formula, and Its Phytochemical Contents and Anti-inflammatory Activity

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Abstract

Ya Kae Fok Buam Mueai Khop (YFBM), a traditional Thai formula, has long been used to relieve pain, aches and swelling, yet lacks substantial research evidence to support its use. This study aimed to investigate the phytochemical contents, anti-inflammatory activity and development of a dosage form for YFBM. The formula comprises *Crateva adansonii* DC., *Zingiber officinale* Roscoe., *Piper retrofractum* Vahl., *Putranjiva roxburghii* Wall., and *Piper nigrum* L. extracts were prepared by maceration with 95% ethanol and decoction with distilled water. Phytochemical contents, including total phenolics, flavonoids and triterpenoids, were measured using the Folin-Ciocalteu method, aluminum chloride method and vanillin-perchloric acid method, respectively. Cell viability of the extracts was assessed using the WST-1 assay. The inhibitory effects of the extracts on lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in HDFn and C2C12 cells were examined. The ethanolic extract had the highest total phenolic, total flavonoid and total triterpenoid contents compared to the aqueous extract. Results indicated that both aqueous (62.5-500 µg/mL) and ethanolic extracts (62.5-125 µg/mL) were non-toxic to C2C12 cells, and similarly, aqueous (62.5-1,000 µg/mL) and ethanolic extracts (62.5–250 µg/mL) were non-toxic to HDFn cells. The ethanolic extract demonstrated superior inhibition of PGE_2 and NO production in LPS-induced C2C12 and HDFn cells compared to the aqueous extract. The ethanolic extract was developed into four spray formulations and tested for physical and chemical stability at 27±2°C and 40±2°C over 45 days. The spray formulations exhibited a greenish-yellow, clear appearance without sedimentation, with spray formula 2 maintaining consistent pH over 45 days at 27±2°C. These findings suggest that the ethanolic extract of YFBM possesses significant phytochemical profiles and anti-inflammatory properties, supporting its traditional use and potential for pharmaceutical spray development.

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Introduction

Inflammation is a protective or healing response to tissue injury in the body and can be caused by different stimuli such as bacterial infection, physical and chemical factors, immunological reactions and tissue damage (Adegbaju et al., 2020; Ansar & Ghosh, 2016). There are four primary indicators of inflammation: pain, redness, heat or warmness and swelling (Verma, 2016). Inflammation is either acute or chronic inflammation. Acute inflammation may be an initial response of the body to harmful stimuli. In chronic inflammation, the inflammatory response is out of proportion resulting in damage to the body. Cyclooxygenase (COX) is the key enzymes in the synthesis of prostaglandins, which are involved in inflammation, pain and platelet aggregation (Pilotto et al., 2010). Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NOS), which regulates several physiological functions. NO is considered as a pro-inflammatory mediator that induces inflammation due to overproduction of abnormal situations (Sharma et al., 2007). Uncontrolled inflammatory response is the main cause of a vast continuum of disorders including allergies, cardiovascular dysfunctions, metabolic syndrome, cancer and autoimmune diseases (Ghasemian et al., 2016). Steroidal and non-steroidal anti-inflammatory drugs (SAIDs and NSAIDs, respectively) are currently the most widely used drugs in the treatment of acute inflammatory disorders (Su et al., 2011). However, these drugs were often associated with adverse effects such as ulceration, perforation, gastric irritation, haematochezia, angioedema, hepatic failure, headache, hemolytic anemia, hyperglycemia, osteoporosis and immunodeficiency-related problems (Bagad et al., 2013).

Herbal medicines are of great importance in primary healthcare medicine. Thai traditional medicine used the Ya Kae Fok Buam Mueai Khop (YFBM), a formula in Folk Formulary, for relieving pain, fatigue and swelling. The formula consists of five plants that are ground into a powder, including *Crateva adansonii* DC., *Zingiber officinale* Roscoe., *Piper retrofractum* Vahl., *Putranjiva roxburghii* Wall., and *Piper nigrum* L. YFBM formula powder is dissolved in alcohol or kaffir lime juice, then applied to affected skin 2-3 times a day (Department of Thai Traditional and Alternative Medicine, 2021). An *in vitro* study on the pharmacological properties and phytochemical constituents of herbs in the YFBM formula identified a marker compound lupeol from *Crateva adansonii* DC., which showed an anti-inflammatory effect against COX-2, MPO, TNFα, IL1β and IL6 (Thirumalaisamy et al., 2020). *Zingiber officinale* Roscoe. is abundant in active constituents, such as phenolic and terpene compounds (Mao et al., 2019). The extracts of rhizome and callus from *Zingiber officinale* Roscoe. were shown to significantly ($P < 0.05$) suppress the LPS-induced production of TNF-α, IL-1 and IL-6 in a dose-dependent manner (Ali et al., 2019). *Piper retrofractum* Vahl. was reported to contain amides, alkyl glycosides, phenylpropanoids, glycosides and lignans (Salleh & Ahmad, 2020). *Piper retrofractum* Vahl. extract possesses an anti-inflammatory property by reducing proinflammatory cytokine production through inhibition of the NF-κB signaling pathway (Lallo et al., 2023). Various parts of *Putranjiva roxburghii* Wall. contain many glucoputranjivin, glycosides glucojiaputin, glucococchlearin, saponins, glucocleomin, flavonoids and triterpenes (Kumar, 2020). The extract of *Putranjiva roxburghii* Wall. exhibited moderate inhibitory activity against inflammation in carrageenin-induced paw edema in rats (Reanmongkol et al., 2009). Piperine from *Piper nigrum* L. inhibited the expression of IL6 and MMP13 and reduced the production of PGE_2 in a dose-dependent manner at concentrations of 10 to 100 μg/mL. Moreover, Piperine significantly reduced nociceptive and arthritic symptoms in rats (Bang et al., 2009). However, there is no scientific research available on the biological activity or chemical profile of the YFBM formula. Therefore, the aim of this study was to investigate the phytochemical contents and anti-inflammatory properties of the YFBM formula. Furthermore, the YFBM formula was developed into a spray and the stability of the product was evaluated.

Materials and methods

1. Materials and chemicals

Quercetin, gallic acid, aluminium chloride, lipopolysaccharide (LPS) and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich USA. Folin-Ciocalteu reagent, acetic acid, ursolic acid, perchloric acid, ethanol and methanol were purchased from Merck, Germany. Vanillin was purchased from Carlo Erba, Italy. 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1), penicillin streptomycin and fetal bovine serum (FBS) were purchased from Gibco, USA. Dulbecco's Modified Eagle Medium (DMEM) was purchased from ATCC, USA.

Prostaglandin E_2 ELISA kit was purchased from Cayman Chemical, USA. Nitric oxide assay kit was purchased from Thermo Fisher, Austria. Tween 20 was purchased from Krungthepchemi Co., Ltd., Thailand. Borneol, camphor, mental, glycerin, eucalyptus oil and methyl salicylate were purchased from Hong Huat Co., Ltd. Thailand.

2. Preparation of sample herbs

The Ya Kae Fok Buam Mueai Khop (YFBM) formula consists of five types of herbs. The leaves of *Crateva adansonii* DC., the rhizomes of *Zingiber officinale* Roscoe., the fruits of *Piper retrofractum* Vahl., the leaves of *Putranjiva roxburghii* Wall. and the seeds of *Piper nigrum* L. were obtained from Vejpong Pharmacy (Hock An Tang) Company Limited (Bangkok, Thailand). All herb samples were cleaned with water and dried at 60°C for 48 hr in a hot air oven. All dried samples were pulverized into fine powder using a stainless-steel blender and passed through a sieve (50 mesh number). The powder (60 g) of each herb sample was mixed for further extraction.

3. Preparation of crude extracts

For an aqueous extract, 300 g of the powder from the YFBM formula was mixed with 6,000 mL of boiling water and stirred using a magnetic stirrer for 15 min. Then the extract was filtered over Whatman No.1 paper. The aqueous extract was dried overnight in a freeze dryer. For an ethanolic extract, 300 g of the powder from the YFBM formula was macerated in 3,000 mL of 95% ethanol at room temperature for 3 days, then filtered. The ethanol solvent was removed at 40°C under reduced pressure by rotary evaporator to yield crude extract. The dried extracts were stored at -20°C prior to phytochemical characterization and bioassay (Gülçin, 2005).

4. Determination of phytochemical contents of YFBM formula extracts

4.1 Determination of total phenolic content

The total phenolic content in the YFBM formula extracts was assessed using the Folin-Ciocalteu assay (Kanlayavattanakul et al., 2012). A gallic acid standard solution (25-200 µg/mL) was prepared in distilled water. One milligram per milliliter of each extract solution was prepared in ethanol. Either gallic acid standard (25 µL) or the YFBM formula extracts (25 µL) were mixed with 25 µL of 2N Folin-Ciocalteu reagent and 75 µL of distilled water. After the solutions were mixed and left for 8 min, 100 µL of 20% sodium carbonate was added. The solution was allowed to stand for 90 min in the dark at room temperature and the absorbance was measured at the wavelength of 765 nm using a microplate reader (Ensight3400; Perkin Elmer, USA). Total phenolic contents of extracts were expressed as gram gallic acid equivalent (GAE) per 100 g of extract. Each sample extract and standard were measured in triplicate (mean±SD).

4.2 Determination of total flavonoid content

The aluminium chloride method was used to determine total flavonoid in the YFBM formula extracts (Chewchinda et al., 2019). A quercetin standard solution (10-60 µg/mL) was prepared in methanol. Five milligrams per milliliter of each extract solution was prepared with ethanol. In each reaction, 100 µL of either quercetin standard or extract solutions was added into the mixture 100 µL of 2% aluminium chloride solution. After mixing, the reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at the wavelength of 415 nm using a microplate reader. Total flavonoid contents of extracts were expressed as gram quercetin equivalent (QE) per 100 g of extract. Each sample extract and standard were measured in triplicate (mean±SD).

4.3 Determination of total triterpenoid content

The total triterpenoid content of the YFBM formula extracts was determined by vanillin-perchloric acid method (Luo et al., 2021). Ursolic acid standard solution (200 - 1,000 µg/mL) was prepared in methanol. Five milligrams per milliliter of each extract was dissolved in 1 mL of methanol and then 100 µL of each sample solution was mixed with 150 µL of a 5% vanillin-glacial acetic acid solution, followed by 500 µL of perchloric acid. Sample solutions were heated for 45 min at 60°C and cooled in an ice water bath to the ambient temperature. After that, 2.25 mL of glacial acetic acid was added and the absorbance of the sample solutions was measured at the wavelength of 450 nm using a UV-visible spectrophotometer (UV-1280; Shimadzu, Japan). Total terpene contents of extracts were expressed as gram ursolic acid equivalent (UE) per 100 g of extract. Each sample extract and standard were measured in triplicate (mean±SD).

5. Determination of anti-inflammatory activity of YFBM formula extracts

5.1 Cell cultures

Neonatal human dermal fibroblast (HDFn, ATCC® PCS-201-010™) and mouse myoblast cell line (C2C12, ATCC® CRL-1502) were purchased from the American Type Culture Collection (ATCC), USA. Both

cell lines were cultured and maintained in Dulbecco's modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin streptomycin and stored at 37°C with 5% CO_2 in a humidified incubator. All cell cultures were subcultured every 3 days and maintained in a fresh specified media. All steps of cell culturing and maintaining were conducted in aseptic condition using the biological safety cabinet.

5.2 Cell viability assay

Cell viability of the YFBM formula extracts was investigated using 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Praphasawat et al., 2016). The HDFn and C2C12 cell lines at density of $2x10^4$ cells/mL were seeded onto 96 well plate and incubated at 37°C under 5% CO_2 in a humidified incubator for 24 hr. After the incubation, the media was removed and replaced with 200 µL of fresh media containing extracts (62.5 - 2,000 µg/mL). For the control group, cells were not treated with extracts. Cell cultures were further incubated at 37°C under 5% CO_2 in a humidified incubator for 24 hr. By the end of treatments, the cells were washed once with phosphate buffer saline before adding of 100 µL WST-1 solution to each well. The plates were kept in darkness at 37°C with 5% CO_2 in a humidified incubator for 30 min. The absorbance was measured at 450 nm using a microplate reader. The percentage viability of cell was calculated as the following equation:

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

5.3 Measurement of PGE_2 production

PGE_2 concentration was measured using a Prostaglandin E_2 ELISA kit (No cat. 0670772) according to the manufacturer's protocols. The cell lines were seeded at a density of 2×10^5 cells/mL in 24-well tissue culture plates. The aqueous extract was prepared with DMEM at the concentration of 500 µg/mL for treating C2C12 and HDFn cells. The ethanolic extract was prepared with DMEM at concentrations of 125 and 250 µg/mL for treating C2C12 and HDFn cell lines, respectively. Then, cells were treated with various concentrations of extracts for 24 hr and stimulated with LPS at a concentration of 10 µg/mL for 24 hr. A positive control was induced cells with LPS (10 µg/mL) without treatment, while the negative control was normal cells without LPS induction. The medium was collected in microcentrifuge tubes and centrifuged (2,000 × g, 5 min, 4°C). The supernatants (100 µL) were transferred into a 96-well ELISA plate. After that, 50 µL of PGE_2-AP conjugate and PGE_2-AP antibody were added to each well, then placed in an orbital shaker for 2 hr at room temperature. Plates were washed using 400 µL wash buffer three times, then 100 µL of the pNpp substrate solution was added into each well, followed by incubation for 1 hr. Stop solution of 50 µL was added into each well, and then the absorbance was measured using a microplate reader at a wavelength of 450 nm (Kang et al., 2016).

5.4 Measurement of NO production

The nitrite associated with NO production was determined using the Nitric Oxide Assay kit (No cat. 346290-001) protocol. The cell lines (2×10^5 cells/mL) were seeded in 24-well tissue culture plates. The aqueous extract was prepared with DMEM at the concentration of 500 µg/mL for treating C2C12 and HDFn cells. The ethanolic extract was prepared with DMEM at concentrations of 125 and 250 µg/mL for treating C2C12 and HDFn cell lines, respectively. The cells were treated with various concentrations of extracts and incubated for 24 hr. After that, LPS (10 µg/mL) was added then further incubated for 24 hr. A positive control was induced cells with LPS (10 µg/mL) without treatment, while the negative control was normal cells without LPS induction. Cell culture supernatants were removed by centrifugation. The supernatants (85 µL) were transferred into a 96-well ELISA plate. Five microliters of nitrate reductase and enzyme cofactor were added to each well, then incubated at room temperature for 1 hr. Enhancer solution (5 µL) was added to each well and incubated at room temperature for 10 min. Approximately 50 µL of Griess reagents R1 and R2 were added to each well. The absorbance was measured at a wavelength of 540 nm using a microplate reader (Junlatat et al., 2022).

6. Formulation of spray from YFBM formula extract

6.1 Preparation of spray from YFBM formula extract

The spray was prepared using the simple solution method. For formulas 1 and 2, the YFBM formula extract was dissolved in ethanol. Then, borneol, camphor and mental were thoroughly mixed and added to the YFBM formula extract. Then, glycerin, tween 20, eucalyptus oil and distilled water were added and mixed until the solution became homogeneous. For formulas 3 and 4, borneol, camphor and mental were mixed until the solution reached homogeneity. The YFBM formula extract was dissolved in solution and then, eucalyptus oil and methyl salicylate were added and mixed well. A summary of all formulas are shown in Table 1.

Table 1 Ingredients of spray from the YFBM formula extract

Ingredients	Formula 1 (%)	Formula 2 (%)	Formula 3 (%)	Formula 4 (%)
YFBM formula extract	0.0125	0.0125	0.0125	0.0125
Borneol	10	10	10	10
Camphor	10	10	10	10
Mental	10	15	10	15
Tween 20	2	2	-	-
Ethanol	40	40	-	-
Glycerin	16	20	-	-
Eucalyptus oil	0.3	0.3	0.3	0.3
DI water	q.s.100	q.s.100	-	-
Methyl salicylate	-	-	q.s.100	q.s.100

6.2 Stability test of spray from YFBM formula extract

The stability test of the four spray formulas was carried out under storage at 40±2°C and 27±2°C. Samples were analyzed at the initial time (t_0) and 15, 30 and 45 days after exposure to the atmospheric conditions described above. Parameters evaluated were pH, color and sedimentation. The pH was determined using a pH meter (Proline B210, Germany). The color was analyzed using the Chroma meter CR-400 colorimeter (Konica Minotta, Switzerland). The color meter was calibrated using a white plate CR-A43(y = 85.70, x = 0.3177 and y = 0.3340) and assessed using the DP mode. The analyzed color parameters lightness were L* (0 = black to 100 = white), a* (greenness (-) to redness (+)) and b* (blueness (-) to yellowness (+)). Sedimentation was determined using a centrifuge machine (Hettich, Germany) at 3,000 rpm for 5 min. The measurement of pH, color and sedimentation of each formulation was done in triplicate (mean±SD) (Kartini et al., 2017; Rezagholizade-shirvan et al., 2023).

7. Statistical analysis

Experimental data were reported as means±standard deviation (SD) and expressed as significant differences among the mean values at the 0.05 level. The phytochemical contents of YFBM formula extracts were compared using an independent samples t- test. The anti-inflammatory activity and percentage cell viability of YFBM formula extracts were compared using a one-way ANOVA. The pH and color values of the spray from YFBM formula extract were analyzed by repeated-measures ANOVA.

Results and discussion

1. Preparation of crude extracts

The results revealed that the yield percentages of aqueous extract (17.45% w/w) were higher than ethanolic extract (8.64% w/w) on dry weight bases. However, good yield in aqueous extract might be due to the fact that most of the phytochemicals bear electronegative functional groups which make the compound hydrophilic in nature (Abbas et al., 2021). In this study, the YFBM formula was extracted with either ethanol or water and these solvents are often recommended for extraction because of their difference in polarity, enabling the extraction of different compounds. In addition, they are safe for human consumption. Such extracts could be safely introduced into food products without risking an unacceptable level of hazardous solvent residues (Zhang et al., 2007; Waszkowiak et al., 2015)

2. Determination of phytochemical contents of the YFBM formula extracts

Table 2 shows the total phenolic, flavonoid and triterpenoid contents of the aqueous and ethanolic extracts of the YFBM formula. Total phenolic content in the aqueous and ethanolic extracts was determined from a linear gallic acid standard curve. This result suggested that the ethanolic extract provided a significantly higher total phenolic content than the aqueous extract, with a total phenolic content of 28.63±1.20 and 22.25±1.28 g gallic acid equivalent per 100 g extract, respectively. The total flavonoid content of extracts was determined by the aluminum colorimetric method using quercetin as the standard. The result indicated that the ethanolic extract had a higher total flavonoid content (0.75±0.26 g QE/100 g extract) than the aqueous extract (0.63±0.14 g QE/100 g extract). Total triterpenoid content in the aqueous extract and ethanolic extract was determined from a linear usoric acid standard curve. The highest content of total triterpenoid was detected in the ethanolic extract with statistical significance (35.52±0.06 g UE/100 g of extract). Altogether, these results showed that the ethanolic extract had higher total phenolic, flavonoid and triterpenoid contents than the aqueous extract. This indicated that the type of extraction solvent and its polarity may have a significant impact on the level of the extracted active compound (Thouri et al., 2017). Pandey

Table 2 Total phenolic, flavonoid and triterpenoid contents of the YFBM formula extracts

Samples	Total phenolic content (g GAE/100 g of extract)	Total flavonoid content^{ns} (g QE/100 g of extract)	Total triterpenoid content (g UE/100 g of extract)
Aqueous extract	22.25 ± 1.28^{b}	0.63 ± 0.14	4.11 ± 0.01^{b}
Ethanolic extract	28.63 ± 1.20^{a}	0.75 ± 0.26	35.52 ± 0.06^{a}

Remark: Data are presented as mean ± standard deviation. Different superscript letters (a-b) in the same column are significant differences among the means of groups (P-value < 0.05); ns indicates not significant (P-value > 0.05).

and Tripathi (2014) also reported that ethanol was a more effective solvent for the extraction of active compounds from plants including tannins, polyphenols, polyacetylenes, flavonols, terpenoids, sterols and alkaloids.

3. Determination of anti-inflammatory activity of YFBM formula extracts

3.1 Cell viability assay

Cell viability was measured by WST-1 assay based on the conversion of slightly red tetrazolium salt to form a dark red formazan product. The results indicated that the viability of C2C12 cells of aqueous extract in concentrations of 62.5, 125, 250 and 500 µg/mL was over 90% in all treatments compared to the control (C2C12 cells without treatment), while that of the ethanolic extract in concentrations of 62.5 and 125 µg/mL was 102.12±2.51 and 82.04±0.82, respectively (Table 3). According to ISO 10993-5, percentages of cell viability above 80% are considered non-cytotoxic (International Organization for Standardization (ISO 10993-5:2009), 2009). The result showed that the aqueous extract (62.5–500 µg/mL) and ethanolic extract (62.5–125 µg/mL) in the concentration used were nontoxic to the C2C12 cells. Therefore, the concentrations of aqueous extract and ethanolic extract used in the inhibition of PGE_2 and NO production experiments in C2C12 cells were prepared in the interval of 500 and 125 µg/mL, respectively. Table 4 shows the percent cell viability of HDFn cells for the aqueous extract and ethanolic extract. The concentrations of 62.5, 125, 250, 500 and 1,000 µg/mL of aqueous extract showed non-toxicity on the HDFn cells when compared to the control (HDFn cells without treatment), while the ethanolic extract in concentrations of 62.5, 125 and 250 µg/mL showed non-cytotoxicity with a percentage viability of HDFn cells higher than 90%. Therefore, the concentrations of aqueous extract and ethanolic extract used in the inhibition of PGE_2 and NO production experiments in HDFn cells were prepared in the interval of 500 and 250 µg/mL, respectively.

3.2 Measurement of PGE_2 production

The effects of aqueous extract and ethanolic extract of the YFBM formula on LPS-induced secretion of PGE_2 were measured based on comparison between treatment and positive control (positive control-treatment/ positive control × 100). A positive control was induced cells with LPS without treatment, while the negative control was normal cells without LPS induction. Quantification of PGE_2 revealed that the aqueous extract and ethanolic extract had an inhibition effect toward the production of PGE_2 in LPS-induced C2C12 cells and HDFn cells. Among the treatments in C2C12 cells, the ethanolic extract had a higher PGE_2 inhibition activity than the aqueous extract. Both extracts had significantly lower PGE_2 concentrations than the positive control. For the treatments in HDFn cells, the ethanolic extract had higher PGE_2 inhibition activity than the aqueous extract. The ethanolic extract had significantly lower PGE_2 concentration than the positive control. The result suggested that the ethanolic extract had the ability to inhibit PGE_2 production in inflammation (Table 5).

Table 3 Percentage cell viability of C2C12 cells exposed to control, aqueous extract and ethanolic extract of the YFBM formula

Samples	% Cell viability					
	62.5 µg/mL^{ns}	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	2000 µg/mL
Control	100.64±0.69	100.64±0.69^{a}	100.64±0.69^{a}	100.64±0.69^{a}	100.64±0.69^{a}	100.64±0.69^{a}
Aqueous extract	100.50±1.20	99.02±0.94^{a}	97.88±2.72^{a}	91.81±1.49^{b}	66.22±2.04^{b}	34.32±1.28^{b}
Ethanolic extract	102.12±2.51	82.04±0.82^{b}	14.62±0.99^{b}	15.53±0.90^{c}	15.49±1.16^{c}	18.82±0.07^{c}

Remark: Data are presented as mean ± standard deviation. Different superscript letters (a-c) in the same column are significant differences among the means of groups (P-value < 0.05); ns indicates not significant (P-value > 0.05).

Table 4 Percentage cell viability of HDFn cells exposed to control, aqueous extract and ethanolic extract of YFBM formula

Samples	% Cell viability					
	62.5 µg/mL	125 µg/mL^{ns}	250 µg/mL	500 µg/mL	1000 µg/mL	2000 µg/mL
Control	100.00±0.00^{b}	100.00±0.00	100.00±0.01^{a}	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}
Aqueous extract	98.07±2.17^{b}	94.27±1.99	93.64±1.87^{ab}	92.03±1.52^{a}	80.15±1.63^{b}	52.82±1.48^{b}
Ethanolic extract	113.15±1.72^{a}	109.86±1.19	91.22±1.73^{b}	41.02±1.84^{b}	34.07±1.18^{c}	28.13±0.98^{c}

Remark: Data are presented as mean ± standard deviation. Different superscript letters (a-c) in the same column are significant differences among the means of groups (P-value < 0.05); ns indicates not significant (P-value > 0.05).

3.3 Measurement of NO production

In the present study, aqueous extract and ethanolic extract of the YFBM formula were evaluated for inhibition of NO production in the LPS-stimulated cells compared to the positive control, which is untreated LPS-induced cells (Table 6). The results showed that the ethanolic extract had a higher NO inhibition activity in C2C12 cells than the aqueous extract. However, both aqueous extract and ethanolic extract had significantly lower NO concentrations than that of the positive control (LPS-induced C2C12 cells without treatment). Among the treatments in HDFn cells, the ethanolic extract had a significantly higher NO inhibition activity than that of the aqueous extract, while the ethanolic extract had significantly lower NO concentrations than those of the aqueous extract and positive control. This indicated that the ethanolic extract was able to inhibit NO production in LPS-induced cells.

Several research studies have investigated the anti-inflammatory effects of herbs in the YFBM formula. Piperine from *Piper nigrum* L. has an anti-inflammatory property against NO and PGE_2 production (Pei et al., 2020). The leaf extracts of *Crateva adansonii* DC. contained bioactive phytoconstituents such as polyphenols (tannins and flavonoids), steroids, alkaloids, coumarin, carbohydrates and terpenoids. Three phytocompounds of *Crateva adansonii* DC., phytol, 1-Hexyl-2-Nitrohexane and 2-Isopropyl-5-Methylcyclohexyl 3-(1-(4-Chlorophenyl)-3-Oxobutyl)-Coumarin-4-Yl Carbonate, possessed an anti-inflammatory property against four inflammatory receptor targets of COX-2, TNFα, IL-1β and IL-6 through an *in silico* study (Thirumalaisamy et al., 2018). 12-dehydrogingerdione (DHGD) of *Zingiber officinale* Roscoe. was shown to have an anti-inflammatory activity. The compound in *Zingiber officinale* Roscoe., 6-shogaol, inhibited LPS-induced interleukin (IL)-6, tumor necrosis factor (TNF)-α, prostaglandin (PGE_2), nitric oxide (NO), inducible NO synthase (iNOS) and cyclooxygenase (COX-2) without interfering with COX-1 in cultured microglial cells (Zhao et al., 2019). The ethanolic extract of *Piper retrofractum* Vahl. exhibited high NO production inhibition in RAW 264.7 cells with an IC_{50} of 25.9±2.5 µg/mL (Kakatum et al., 2012). *Putranjiva roxburghii* Wall. extract at 100 µg/mL exhibited NO production inhibition in RAW 264.7 cells (Sudha Bai & Sarath, 2019). The results from these studies are congruent with the results of this study. Furthermore, the extracts of the YFBM formula exhibited the anti-inflammatory activity against the production of NO and PGE_2 in cells. It was reported that phytochemical compounds are present in herbal medicines, such as alkaloids, flavonoids, phenolic compounds, glycosides and terpenoids and are effective anti-inflammatory agents (Manivannan & Johnson, 2020). In response to inflammatory stimuli, microglia release proinflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE_2) through the activation of nuclear factor (NF)-κB, which normally switches on a protective response in the central nervous system (Boje & Arora, 1992). PGE_2 levels are related to muscular pain in patients with fibromyalgia, while NO has pro-inflammatory properties and causes damage to skeletal muscle (Sakurai et al., 2013; Hedenberg-Magnusson et al., 2001). The result indicated that the phytochemical constituents of the extract from the YFBM formula possess an anti-inflammatory activity. Consequently, data obtained from this study support the use of the YFBM formula for reducing muscle pain, aching and swelling due to its potent inhibitory activity on NO and PGE_2 produced in cells.

Table 5 Effect of aqueous extract and ethanolic extract of the YFBM formula on PGE_2 in LPS-stimulated cells

Samples	C2C12 cells		HDFn cells	
	PGE_2 level (pg/mL)	PGE_2 inhibition activity (%)	PGE_2 level (pg/mL)	PGE_2 inhibition activity (%)
Negative control	20.90±1.92^{c}	-	24.36±0.92^{c}	-
Positive control	72.77±0.46^{a}	0.00±0.00^{c}	62.18±0.19^{a}	0.00±0.00^{c}
Aqueous extract	36.67±0.55^{b}	49.61±1.07^{b}	54.89±0.55^{b}	11.58±0.13^{b}
Ethanolic extract	24.73±1.03^{c}	66.09±1.93^{a}	52.07±1.73^{b}	16.07±0.21^{a}

Remark: Data are presented as mean ± standard deviation. Different superscript letters (a-c) in the same column are significant differences among the means of groups (P-value < 0.05).

Table 6 Effect of aqueous extract and ethanolic extract of the YFBM formula on NO production in LPS-stimulated cells

Samples	C2C12 cells		HDFn cells	
	NO level (µM/ml)	NO inhibition activity (%)	NO level (µM/ml)	NO inhibition activity (%)
N Negative control	2.36±0.15^{c}	-	1.34±0.29^{c}	-
Positive control	5.46±0.25^{a}	0.00±0.00^{c}	5.21±0.49^{a}	0.00±0.00^{c}
Aqueous extract	3.57±0.41^{b}	32.12±1.42^{b}	4.50±0.38^{a}	13.41±1.02^{b}
Ethanolic extract	3.18±0.27^{b}	44.70±0.97^{a}	3.40±0.17^{b}	31.14±1.66^{a}

Remark: Data are presented as mean±standard deviation. Inhibitory activity over positive control was measured based on comparison between treatment and positive control (positive control-treatment/positive control×100). Different superscript letters (a-c) in the same column are significant differences among the means of groups (P-value < 0.05).

4. Formulation of spray from YFBM formula extract

The results suggested that the ethanolic extract contained high phytochemical content and has high anti-inflammatory activity. Thus, the ethanolic extract of the YFBM formula was selected to be developed into four spray formulations. Ethanol extract of 0.0125% was used to prepare 100 mL of spray, which was calculated from the concentration used in the cell (125 µg/mL). This concentration was not toxic to cells and was anti-inflammatory. In addition, 0.0125% ethanol extract could be solubilized and homogenized in the formulation. However, when applying a substance topically, especially on the skin, its absorption and penetration capabilities become critical. Active substance permeation across the skin is a complex process and the main steps are the diffusion of the substance within the topical formulation, the release from it and the penetration into the skin. Because of the complex and non-homogeneous skin structure, the description and modelling of active substance permeation are difficult (Butkeviciute et.al, 2022). Even though the extract might be active at a certain concentration in vitro (in cell cultures), it may not exhibit the same efficacy when applied to the skin due to barriers like the skin's outer layer (stratum corneum). Therefore, the concentration needed adjustment to ensure sufficient absorption and penetration into the skin to exert its effectiveness. The stability of four spray formulas was tested at 27±2°C and 40±2°C for 0, 15, 30 and 45 days. The results showed that the four spray formulas were still clear with no sediment in both conditions of 27±2°C and 40±2°C. The results from the color parameter analysis of four spray formulas at 27±2°C showed that the lightness (L*) values ranged from 24.31±0.04 to 27.64±0.03, the a* value ranged from -0.17±0.62 to -0.77±0.02 and the b* value ranged from 1.78±0.04 to 3.46±0.01, as shown in Table 7. At 40±2°C, the color parameter analysis of four spray formulas showed that the lightness (L*) values ranged from 23.77±0.03 to 27.55±0.06, the a* value ranged from -0.44±0.01 to -0.87±0.03 and the b* value ranged from 2.13±0.02 to 3.35±0.01 (Table 7). The results indicated that the four spray formulas had the same green (-a*) and yellow (+b*) colors. The lightness (L*) value of all spray formulas was in the range of white, which shows that all spray formulas were clear. The pH values of the four spray formulas are shown in Table 8. At 27±2°C, four spray formulas had pH values ranging from 4.92±0.08 to 6.35±0.07, while those at 40±2°C had the pH values ranged from 4.79±0.03 to 6.30±0.66 on day 0 to 45. However, when the pH was tested at day 45 at 27±2°C, it was found that only spray formula 2 showed no significant difference when compared to those at day 0. Therefore, it was concluded that spray formula 2 could be stored at 27±2°C. At day 45, the pH of spray formula 2 was 5.91±0.20, which corresponds to skin pH values. It is important that a topical spray formulation has the pH range of 4 – 6 to allow for better absorption (Lukić et al., 2021). Nevertheless, spray formulas 3 and 4 may contain high methyl salicylates, which may cause irritant or allergic contact dermatitis and anaphylactic reactions (Chan, 1996). Further research is needed to determine the stability of the spray under low temperature conditions as different temperature conditions will impact on the stability, which directly relates to the length of shelf life that can be recommended (Magari, 2003). In addition, it is also essential for microbiological testing to be performed to determine the microbial shelf life. This involves monitoring the growth and activity of microorganisms, such as bacteria, yeasts and molds, to ensure that their populations remain controlled and do not reach levels that compromise the safety or quality of the product. An accurate estimation of microbial shelf life is crucial for ensuring the safety of products and preventing the onset of microbial-related deterioration (Tarlak, 2023).

Table 7 Color parameters of the four spray formulas from the YFBM formula extract

Temperature	Days	Formula 1			Formula 2			Formula 3			Formula 4		
		L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
27±2°C	0	24.76±0.02^{a}	-0.45±0.03^{a}	1.78±0.04^{c}	24.75±0.24^{ns}	-0.17±0.62^{ns}	2.37±0.01^{c}	26.51±0.04^{b}	-0.76±0.02^{ns}	3.46±0.01^{a}	27.31±0.06^{bc}	-0.69±0.03^{a}	2.28±0.02^{b}
	15	24.52±0.04^{b}	-0.64±0.04^{b}	2.39±0.02^{b}	24.80±0.12^{ns}	-0.66±0.03^{ns}	2.98±0.08^{a}	26.49±0.01^{b}	-0.77±0.02^{ns}	3.42±0.03^{a}	27.12±0.05^{bd}	-0.76±0.01^{ab}	2.49±0.04^{a}
	30	24.31±0.04^{c}	-0.57±0.25^{b}	2.69±0.02^{a}	24.82±0.25^{ns}	-0.60±0.03^{ns}	2.65±0.13^{bc}	26.92±0.04^{a}	-0.73±0.01^{ns}	3.17±0.03^{b}	27.45±0.18^{ab}	-0.74±0.03^{ab}	2.50±0.08^{a}
	45	24.63±0.06^{ab}	-0.65±0.02^{b}	2.69±0.05^{a}	24.80±0.15^{ns}	-0.64±0.05^{ns}	2.68±0.03^{b}	26.50±0.02^{b}	-0.76±0.03^{ns}	3.44±0.04^{a}	27.64±0.03^{a}	-0.73±0.03^{b}	2.47±0.04^{a}
40±2°C	0	24.73±0.03^{b}	-0.55±0.03^{b}	2.36±0.02^{a}	23.77±0.03^{b}	-0.72±0.01^{b}	3.35±0.01^{a}	27.32±0.10^{a}	-0.76±0.02^{a}	3.00±0.04^{b}	26.69±0.03^{b}	-0.71±0.02^{c}	2.97±0.01^{b}
	15	25.04±0.06^{a}	-0.45±0.03^{a}	2.19±0.01^{b}	24.49±0.14^{a}	-0.60±0.03^{a}	2.72±0.06^{c}	27.41±0.06^{a}	-0.75±0.01^{a}	2.75±0.03^{c}	26.74±0.02^{b}	-0.74±0.02^{bc}	3.00±0.01^{a}
	30	24.55±0.14^{bc}	-0.44±0.01^{a}	2.19±0.05^{ab}	24.36±0.11^{a}	-0.67±0.01^{a}	2.90±0.03^{b}	27.00±0.06^{b}	-0.85±0.03^{b}	3.25±0.04^{a}	27.45±0.03^{a}	-0.62±0.04^{ab}	2.13±0.02^{c}
	45	24.52±0.04^{c}	-0.46±0.03^{ab}	2.22±0.06^{ab}	24.56±0.06^{a}	-0.65±0.04^{ab}	2.92±0.02^{b}	27.08±0.11^{ab}	-0.87±0.03^{b}	3.25±0.05^{a}	27.55±0.06^{a}	-0.60±0.03^{a}	2.15±0.03^{c}

Remark: Data are presented as mean±standard deviation. Different superscript letters (a-d) in the same column at conditions 27±2°C and 40±2°C indicate significant differences among the means of the groups (P-value < 0.05); ns indicates not significant (P-value > 0.05).

Table 8 pH values of the four spray formulas from the YFBM formula extract

Temperature	Formulas	Days			
		0	15	30	45
27±2°C	1	6.35±0.07^{a}	6.23±0.05^{b}	5.80±0.06^{c}	5.82±0.04^{c}
	2^{ns}	6.19±0.04	6.15±0.05	6.04±0.10	5.91±0.20
	3	5.51±0.19^{a}	5.43±0.12^{ab}	5.17± 0.11^{b}	5.04±0.12^{c}
	4	6.09±0.11^{a}	5.67±0.13^{b}	5.18±0.15^{c}	4.92±0.08^{d}
40±2°C	1	6.30±0.11^{a}	6.13±0.02^{a}	5.95±0.27^{b}	5.61±0.07^{c}
	2	6.27±0.03^{a}	6.22±0.05^{a}	6.08±0.08^{b}	5.64±0.12^{c}
	3	6.30±0.66^{a}	5.65±0.12^{b}	4.95±0.23^{c}	4.79±0.22^{d}
	4	5.87±0.12^{a}	5.56±0.08^{b}	5.06±0.10^{c}	4.79±0.03^{d}

Remark: Data are presented as mean±standard deviation. Different superscript letters (a-d) in the same row at conditions 27±2°C and 40±2°C indicate significant differences among the means of the groups (P-value < 0.05); ns indicates not significant (P-value > 0.05).

Conclusion

The ethanolic extract of the YFBM formula is rich in anti-inflammatory compounds such as flavonoid, phenolic and terpene compounds. The extract showed potent inhibitory activity against lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in HDFn and C2C12 cells. Therefore, the ethanolic extract of the YFBM formula has the potential to be developed as an alternative raw material for developing into a spray product for pharmaceutical purposes.

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