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**Bioactivity test and GC-MS analysis of different solvent extracts from *Perilla frutescens* (Linn.) Britton and cosmetic product application for sensitive skin**

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### **Abstract**

The biological activities of petroleum ether (P), 95% ethanol (E) extracts and hydro-distillation (H) of *Perilla frutescens* (Linn.) Britton leaves were evaluated. Total phenolic contents, anti-oxidant activity (DPPH<sup>•</sup> and hydroxyl radical (•OH) scavenging) and the anti-inflammatory effects were determined. The volatile components of the hydro-distillation were determined by GC-MS analysis. The stability of cosmetic products containing the extract and dermal irritancy on human skin were also evaluated. The results suggested that the total phenolic content in E extract was higher than that in P extract. All extracts presented significantly inhibited hydroxyl radical induced deoxyribose degradation with an IC<sub>50</sub> value in the range of 0.045±0.017-0.092±0.024 mg/ml more than Trolox (IC<sub>50</sub>=1.351±0.408 mg/ml). For DPPH assay, E extract showed significantly (p<0.05) higher activity than P extract. The highest ability of inhibition exhibited in E extract was 040E (IC<sub>50</sub>=0.824±0.061 mg/ml) which is also related to GEAC (3.298±0.237 mg/g extract) and TEAC (13.002±0.933 mg/g extract) values. The anti-inflammatory effects of all E extracts at low concentration 0.0625 ug/g were higher activity to NO inhibition than those of all P extracts. GC-MS analysis showed the presence of 2-Hexanoylfuran and 1-Pentanone in all three samples. The irritation on the volunteer's skin was not found. To

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conclude, *P. frutescens* leaves showed antioxidants activity, serving as an anti-inflammatory agent, and volatile compounds (essential oil) which might be a good plant for cosmeceutical products.

**Keywords:** Anti-inflammatory, Antioxidant, Cosmetic product, Skin irritation, Stability test

## 1. Introduction

The excessive production of free radicals leads to oxidative stress. The diseases associated with the reactive oxygen species (ROS) such as superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), alkoxyl ( $RO^\cdot$ ), peroxy (ROO $^\cdot$ ), hydroxyl radical ( $OH^\cdot$ ) as well as reactive nitrogen species (RNS) including nitric oxide ( $NO^\cdot$ ) and peroxynitrite mainly depend on an imbalance between the pro-oxidant and the antioxidant concentration in the body [1, 2]. Free radicals inactivate enzymes and damage important cellular components, causing tissue injury and damage cellular components through covalent binding and lipid peroxidation [3]. Free radicals have been implicated in causation of ailments such as cancer, inflammation, diabetes, hypertension, liver cirrhosis, cardio vascular disease, Alzheimer's, aging and acquired immunodeficiency syndrome [3].

Plants possess a large source of natural antioxidants, phytochemicals promising therapeutic drugs for free radical pathologies which leads to the development of novel drugs for maintenance of human health, prevention and treatment of diseases [1]. Therefore, plants are used to treat the depression, inflammation, bacterial and fungal infections, and tumors. Plants also have the anti-

oxidant, anti-inflammatory, anti-allergic, anti-microbial, and anti-cancer potentials [4, 5]

*Perilla frutescens* (L.) Britton is an annual plant belonging to the mint family *Lamiaceae*, subfamily *Lanoideae* [6] which has been widely used as edible and medicinal plant. It is common in Asian countries for hundreds of years, especially in China, Japan, Korea [7, 8], and Thailand. The common names are Ren in Chinese, Egoma in Japan, Dlaggae in Korea [9] and Nga-mon in Thailand. *P. frutescens* contains other anti-inflammatory polyphenols (apigenin, caffeic acid, and luteoline), the compounds also suppress proallergic immune response [10, 11].

The phenolic compounds that isolated from *P. frutescens* can be expected to possess various biological activities [12]. The main active constituents of perilla leaves include flavonoids, saponins, polysaccharides, amino acids, and trace elements. The flavonoids, existing in many types in plant, have been reported to provide anti-inflammatory and anti-allergic activities by suppressing the proliferation and activity of lymphocytes [10, 13]. Furthermore, rosmarinic acid, one of major polyphenol compounds found in the stems, leaves, and seeds of *P. frutescens* [8], has been known as a highly efficient superoxide radical

scavenger and inhibits epidermal inflammatory responses [12, 14] and shows anti-allergic activity [11, 15].

*P. frutescens* leaves have been properly used for their anti-oxidant, anti-allergic, anti-microbial, anti-inflammatory, anti-tumor, and anti-cancer properties, which implicates that it is a result of their phenolic compounds, rosmarinic acid, flavonoids, essential oil, vitamins, and minerals [16]. So that it might be good active ingredients in cosmetic products.

The present work has been designed to evaluate the total phenolic content, anti-oxidant, and anti-inflammatory activity of the petroleum ether, 95% ethanol extracts and volatile compounds of hydro-distillation by GC-MS which were obtained from *P. frutescens* leaves. Moreover, the application to cosmetic product containing *P. frutescens* leaves extract for sensitive skin will be presented. In addition, this work also investigated the safety assessment of the extract and the cosmetics recipe by skin irritation test.

## 2. Materials and Experiments

### 2.1 Sample collection and extraction

*P. frutescens* seeds were obtained from three locations, Pong District Phayao Province (code: 040), Bo Kluea District Nan Province (code: 112) and Chaloem Phra Kiat District Nan Province (code: 115) which were supplied by Nan Agricultural Research and Development Centre. The seeds were grown in the agricultural area of University of Phayao in June. The voucher

specimens (No.93756 Collector Lapatrada Mungmai 1) were deposited in BGO plant database, The Botanical Garden Organization, Thailand.

After five months of growing, the leaves were harvested; the fresh leaves were extracted by hydro-distillation process (H) to obtain volatile components and then stored at 4 °C until gas chromatography-mass spectroscopy (GC-MS) analysis. *P. frutescens* leaves were dried at 60 °C for 3 h. Then the dried samples were grounded into powder with mortar and extracted with petroleum ether (P) and 95% ethanol (E) overnight at ambient temperature. Both of the extract solutions were filtered through filter paper and evaporated under reduced pressure until dryness. The percentage yield of each extract was calculated.

### 2.2 The phytochemical screening

The extracts which presented the highest of percentage yield were selected to be a representative for screening the bioactive compound group. Their bioactive compound group such as flavonoids, alkaloids, phenolic compounds, tannin, coumarin, saponin including anthraquinones were tested by the chemical test [17, 18].

### 2.3 Determination of total phenolic content

Total phenolic content was determined by the Folin-Ciocalteau assay [1, 10]. Two-hundred-fifty microlitres of the extract were mixed with ethanol (250 µL) and deionized water (1,250 µL). Folin-Ciocalteu reagent (250 µL) was added to the mixture. The mixture was shaken and allowed to stand for 5 min at room temperature. Then 250 µL

of 5% w/v  $\text{Na}_2\text{CO}_3$  solution was added to the mixture. After incubation for 1 h in a dark room at room temperature, the absorbance was measured at 725 nm with an UV-Vis spectrophotometer. The total phenolic content was expressed as mg Gallic acid equivalents antioxidant capacity (GAE; mg Gallic acid/g extract).

#### **2.4 Determination of hydroxyl radical scavenging activity**

Hydroxyl radical scavenging assay was determined using methods described by Awah and Verla [1]. The reaction mixture contained 2.8 mM Deoxyribose, 100  $\mu\text{M}$   $\text{FeCl}_3$ , 100  $\mu\text{M}$  Nitrilotriacetic, 2.8 mM  $\text{H}_2\text{O}_2$  and 10 mM Phosphate buffer, (pH 7.4) in final volume of 1 ml. The mixture was incubated in water bath at 37 °C for 1 h, followed by addition of 1 ml of 1% w/v TBA and 1 ml of 2.8% w/v TCA. The mixture was heated at 100 °C for 15 min, then cooled on ice and measured spectrophotometrically the absorbance of the resulting solution at 532 nm. Trolox as the reference standard. The inhibition of deoxyribose degradation was expressed in term of  $\text{IC}_{50}$  value (mg/ml).

#### **2.5 Determination of antioxidant activity by free radical scavenging activity on 2, 2'-diphenyl-1-picrylhydrazyl (DPPH $^\cdot$ )**

DPPH radical scavenging activity was measured using methods adapted from Duan *et al.* [18]. Eight microliters of each extract with different dilutions were added by 67  $\mu\text{L}$  of 1M

Tris-HCl (pH 7.9) and 800  $\mu\text{L}$  of 130  $\mu\text{M}$  DPPH $^\cdot$ /methanol solution in a 96-well microtiter plate. The mixture was allowed to stand for 20 min in the dark at room temperature. Absorbance of the mixtures was measured at 517 nm with microplate reader. Gallic acid and Trolox as the reference standard. Scavenging ability of each sample was then expressed in terms of its Gallic Acid Equivalent Antioxidant Capacity (GEAC), Trolox Equivalent Antioxidant Capacity (TEAC) and  $\text{IC}_{50}$  value (mg/ml).

#### **2.6 Determination of anti-inflammatory activity**

##### **Cell culture**

Raw 264.7 macrophage cells were cultured in DMEM medium with 10% fetal calf serum containing penicillin and streptomycin at 37 °C in a 5%  $\text{CO}_2$  incubator [19].

##### **2.7 MTT cell viability assay**

Cell viability was assessed using a modified MTT assay [19]. Cells were seeded in a 96-well plate and incubated at 37 °C in a 5%  $\text{CO}_2$  incubator for 24 h. The samples (100  $\mu\text{l}$ ) of different concentrations were added to the cells, then incubated at 37 °C in a 5%  $\text{CO}_2$  incubator. Fifteen microliters of 2 mg/ml MTT solution was added to each well and incubated at 37 °C for 4 h; after incubating for 24 h., Dimethyl sulfoxide (DMSO) (200  $\mu\text{l}$ ) was added to each well. The absorbance was measured at 540 nm and 630 nm by microplate reader. The percentage of viability was calculated.

## 2.8 Quantification of nitric oxide (NO) production

The concentration of NO was determined via Griess reaction [19]. Cells were seeded in a 96-well plate and incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 24 h. Cells were treated with 60 µl of the sample, after that incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 1 h. Then 100 µl of Lipopolysaccharide (LPS) (in DMEM no phenol red) were added to the cells and incubated at 37 °C in a 5% CO<sub>2</sub> incubator. After 24 h, 50 µl of supernatant from each well was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate. After an incubation at room temperature for 15 min, the absorbance was measured at 550 nm using microplate reader and expressed as a percentage of that in control cells.

## 2.9 Gas chromatography-mass spectrometry analysis of volatile components

The sample extracts (H) were analyzed in Thermo Fisher Scientific, GC model: TRACE 1300, MS model: ISQ QD using TG-5ms (5% diphenyl/95% dimethyl polysiloxane; 30 m, 0.25 µm, 0.25 mm). The temperature program used for the analysis was as follows: the injector and detector were set to 240 °C and 350 °C, respectively. The column oven temperature was started with an initial isothermal at 60 °C for 2 min, after which the temperature was increased with 3 °C /min until it reached 280 °C and kept at this temperature for 9.50

min. Helium was used as carrier gas, with a controlled constant flow of 10.0 ml/min.

## 2.10 Preparation of *P. frutescens* leaves cream

We formulated cream base for 3 recipes, then tested the stability in various conditions as room temperature, 4 °C and 45 °C for 1 month and 6 cycles of the heating/cooling condition (45 °C, 48 h alteration with 4 °C, 48 h for 1 cycle). The evaluation of their pH, viscosity (Pas) feel on skin and visually physical changing along with color and smoothness was performed [20].

The best cream base was selected to incorporate with the extract for evaluation of cosmetic product safety. A recipe of cosmetics emulsion O/W is shown as the following: Glyceryl Stearate, Cetyl alcohol, Stearic acid, Polysorbate 60, Glycerine, 1,2-hexanediol caprylhydroxamic acid –Butylene glycol, HDI/Trimethylol hexyllactone crosspolymer silica, Carbomer, Cyclopentasiloxane (and) Dimethiconol, Cyclomethicone, Triethanolamine, and DI water. The test cream was prepared by heating the water and oily phases (70-75 °C) separately, then it was homogenized at 3000 rpm for 5 min and mixed by stirrer to achieve a temperature of 45 °C. *P. frutescens* leaves crude extract (0.2 %) which have highest bioactivity was added into the best cream base. The base cream was prepared without the extract. All samples had the pH values of 5.5 and freshly prepared.

The stability of the test cream was tested in various conditions as room temperature, 4°C and 45 °C for 3 month and 6 cycles of the heating/cooling condition (45 °C, 48 h alteration with 4 °C, 48 h for 1 cycle) and also investigated its pH, viscosity (Pas), feel on skin, visually physical changing along with color, and smoothness [20].

### 2.11 Skin irritation test

The safety assessment was investigated by skin irritation test following the method described by slight modifications Barel and Maibach [21] and OECD [22]. This study had been approved by University of Phayao Human Ethic Committee, Thailand. The approval number was 3/019/58. The skin irritation study was carried out on the upper back of the volunteers by using Finn chamber® on thirty healthy volunteers (n=30), ages between 40-60 years old. The test substances containing *P. frutescens* leaves extract, cream base, cream containing *P. frutescens* leaves extract, 1% w/v sodium lauryl sulfate (SLS) (positive control) and deionized water (negative control), were covered for 48 h. After removal of the patch, any irritating reactions (erythema and edema) were observed at 1, 24 and 48 h. Draize scoring system was used to calculate the primary dermal irritation index (PDII).

### 2.12 Statistical analysis

The results are summarized from three independent experiments and presented as means $\pm$ SD. Significant differences were examined using the analysis of variance (ANOVA) followed by Tukey's test. The correlation coefficient was

measured by Pearson's correlation. The data were evaluated with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results and Discussion

### 3.1 Percentage yields of the extraction of *P. frutescens*

The percentage yields of 040, 112 and 115 of *P. frutescens* leaves extracts by the three different solvents (95% ethanol, petroleum ether, distill water by hydro-distillation) are shown in Table 1. The crude extracts from 95% ethanol showed significantly ( $p<0.05$ ) higher percentage yields (6.22-7.65%) than petroleum ether (0.40-0.47%) and hydro-distillation (0.03%), while not significantly different between plants which could be due to the fact that they were grown in the same cultivation area. The different solubility of the solute in different polarity solvents are expected. The mean percentage yields of the extracts decreased in the order of polarity. This indicated that *P. frutescens* may contain higher polar than the non-polar compounds. The ethanolic extract which presented the highest of percentage yield were selected to screening the bioactive compound group. The result revealed that the extracts 040E, 112E, and 115E similarly presented of the bioactive compound groups of flavonoids, alkaloids, phenolics, and coumarins, whereas only 112E extract showed tannin in the ethanolic extract. The results are presented in Table 2. Flavonoids, alkaloids, tannin, and phenolic compounds were known for their function against free radicals and

act as anti-inflammatory agents due to their high antioxidant activity [23, 24, 25]. Coumarins are a

natural compound were reported as effective anticancer agent [26].

**Table 1** Percentage yield and IC<sub>50</sub> standard equivalent of antioxidant activity and total phenolic content of the ethanol extract (E) and the petroleum ether extract (P) of *P. frutescens* leaves extracts.

Extracts	Total Phenolic content		Hydroxyl radical activity	DPPH			
	GAE			IC <sub>50</sub> (mg/ml)	GEAC mg Gallic acid/g extract	TEAC mg Trolox/g extract	
	% Yield	mg Gallic acid/g extract					
<b>040P</b>	0.41	4.973±0.092 <sup>f</sup>	0.048±0.001 <sup>a</sup>	11.634±0.610 <sup>c</sup>	0.233±0.012 <sup>c</sup>	0.919±0.047 <sup>c</sup>	
<b>112P</b>	0.40	13.601±0.321 <sup>d</sup>	0.050±0.002 <sup>a</sup>	16.349±0.880 <sup>d</sup>	0.166±0.009 <sup>c</sup>	0.654±0.035 <sup>c</sup>	
<b>115P</b>	0.47	10.106±0.252 <sup>e</sup>	0.052±0.002 <sup>a</sup>	7.291±0.658 <sup>b</sup>	0.373±0.032 <sup>c</sup>	1.472±0.126 <sup>c</sup>	
<b>040E</b>	7.65	38.151±0.456 <sup>a</sup>	0.092±0.024 <sup>b</sup>	0.824±0.061 <sup>a</sup>	3.298±0.237 <sup>a</sup>	13.002±0.933 <sup>a</sup>	
<b>112E</b>	6.22	36.151±0.487 <sup>b</sup>	0.045±0.017 <sup>a</sup>	1.180±0.011 <sup>a</sup>	2.294±0.021 <sup>b</sup>	9.045±0.083 <sup>b</sup>	
<b>115E</b>	7.00	33.170±0.258 <sup>c</sup>	0.054±0.004 <sup>a</sup>	1.141±0.032 <sup>a</sup>	2.375±0.068 <sup>b</sup>	9.365±0.267 <sup>b</sup>	
<b>Trolox</b>	-	-	1.351±0.408	-	-	-	

- : not determined

**Table 2** The bioactive compounds of the ethanolic extract of *P. frutescens*

Extracts	Flavonoids	Alkaloids	Phenolic	Tannins	Coumarins	Saponins	Anthraquinons
<b>040E</b>	√	√	√	-	√	-	-
<b>112E</b>	√	√	√	√	√	-	-
<b>115E</b>	√	√	√	-	√	-	-

### 3.2 The total phenolic content

The results of the total phenolic content of *P. frutescens* leaves extracts, including 95% ethanol and petroleum ether extracts, are shown in Table 1. When comparing all extracts, the 95% ethanol extract exhibited the highest total phenolic content whereas the petroleum ether extract had the lowest

total phenolic content. The total phenolic content of 95% ethanol extract from three crude extracts, 040E, 112E and 115E were 38.151±0.456, 36.151±0.487 and 33.170±0.258 mg gallic acid/g extract, respectively; whereas the total phenolic content of the petroleum ether extract of 040P, 112P and 115P were 4.973±0.092, 13.601±0.321 and

10.106±0.252, respectively, as the result shown in Table 1. These results conformed to the data of phenolic compounds that present in the phytochemical screening (Table 2) and indicated that the polar solvents, the more total phenolic contents can be extracted and the total phenolic content in the extract often reflects its antioxidant activity. These results are similar with the reports of Chatha *et. al.* [27] suggested that the yields of the phenolic compounds greatly depended on the solvent polarity.

### 3.3 Anti-hydroxyl radical activity

Hydroxyl radicals (OH<sup>·</sup>) are the major active oxygen species causing oxidation of polyunsaturated fatty acids in enormous cellular and tissue damage [1]. The effect of *P. frutescens* leaves extracts are shown in Table 1, all of the extracts significantly ( $p<0.05$ ) inhibited hydroxyl radical-induced deoxyribose degradation with an IC<sub>50</sub> value in the range of 0.045±0.017-0.092±0.024 mg/ml, demonstrating the superiority of the extracts to the Trolox (IC<sub>50</sub>=1.351±0.408mg/ml). The antioxidant components in the extracts could be acting as chelators of the Fe<sup>3+</sup> ions in the system, thereby preventing them from complexing with the deoxyribose. The observed ability of the extracts to scavenge or inhibit OH<sup>·</sup> radical indicated that the extracts could significantly inhibit lipid peroxidation since OH<sup>·</sup> radicals are highly implicated in peroxidation [1].

### 3.4 DPPH radicals scavenging assay

The results of DPPH free radical scavenging activity of the extract are shown in

Table 1 and compared with Gallic acid and Trolox as references. The results suggested that the ethanol extract showed significantly ( $p<0.05$ ) higher activity than petroleum ether extract. The 040E extract showed the higher DPPH scavenging activity with an IC<sub>50</sub> of 0.824±0.061 mg/ml than 115E (1.141±0.032 mg/ml) and 112E (1.180±0.011 mg/ml), respectively. The ability of DPPH radical scavenger of 040E which is higher than other extracts also related with the GAEC (3.298±0.237 mg Gallic acid/g extract) and TEAC (13.002±0.933 mg Trolox/g extract) values. All data obtained in this study could be a result from the active compound of phenolic and flavonoids in the leaf sample (Table 2). We also observed a positive correlation ( $p<0.05$ ) between the total phenolic content and free radical (DPPH<sup>·</sup>) scavenging efficiencies.

The results revealed that the level of total polyphenol content was well correlated with DPPH radical scavenging antioxidant activity. The extracts possessed antioxidant activities due to a combination of their total phenolic content as reported in other studies [25, 28].

Considering the observed antioxidant potential of the investigated *P. frutescens* leaves extracts and the potent DPPH<sup>·</sup> radical and OH<sup>·</sup> radical scavenging activity, it can be presumed that this extract is able to prevent lipid peroxidation and can further suggest that the extract is a potential therapeutic agent for the control of oxidative and non-oxidative damages caused by reactive oxygen and nitrogen species as indicated by Awah and

Verla [1] that the plant contains certain compounds which are potential antioxidants. The addition of the extracts indicated the good scavenging capacity. The extracts showed substantial antioxidant activity more than Gallic acid and Trolox which were used as control standard antioxidants.

### 3.5 Effects of *P. frutescens* leaves extracts on cell viability

The cytotoxicity of *P. frutescens* leaves extracts was determined in macrophage cells using MTT assay. Cells were treated with various concentrations of the extracts; petroleum ether and ethanol extract, ranging from 0.0625-1 mg/ml. The significant change in percent live cell population was not observed ( $p<0.05$ ) at any concentration of

the treatment when compared with normal cells. The results indicated that the 040P extract at the concentrations 0.0625, 0.125 and 0.25 mg/ml, the 112P, 115P, 040E and 112E extracts at the concentrations 0.0625, 0.125, 0.25 and 0.5 mg/ml, the 115E extract at the concentrations 0.0625 and 0.125 mg/ml had low toxicity and showed cell viability more than 95 percentage. Then, these concentrations had been used for subsequent NO inhibition experiments. Cell viability and cytotoxicity are the most important toxicology indicators. The cytotoxicity screening results of this study showed that the extracts possess no risk of skin irritation and inflammatory effect, however it possess high toxicity potential at high concentration.

**Table 3** The percentage of inhibition effects of *P. frutescens* (L.) leaves extracts on NO production in LPS-stimulated RAW 264.7 cells at various concentrations.

Extracts	% Inhibition						
	Concentration (mg/ml) of <i>P. frutescens</i> (L.) leaf extracts						
	Control	0.015625	0.03125	0.0625	0.125	0.25	0.5
LPS							
<b>040P</b>	0.00±0.00	-	0.08±0.14	3.82±3.38	20.25±9.36*	75.57±10.72*	-
<b>112P</b>	0.00±0.00	-	-	4.89±8.48	11.15±12.37	43.31±8.84*	93.54±4.02*
<b>115P</b>	0.00±0.00	-	-	3.40±5.88	11.33±9.51	33.97±2.51*	74.29±10.19*
<b>040E</b>	0.00±0.00	-	-	85.41±5.25*	93.97±6.29*	95.00±2.33*	98.37±2.82*
<b>112E</b>	0.00±0.00	-	-	78.45±15.12*	94.07±7.60*	95.00±8.56*	95.23±5.15*
<b>115E</b>	0.00±0.00	52.49±8.90*	69.11±15.25*	80.14±14.19*	88.88±12.88*	-	-

RAW 264.7 cells were stimulated with 1  $\mu$ g/mL of LPS. Values are means of three replicate determinations ( $n=3$ ),  $\pm$  standard deviation.

\* Inhibitory values are significantly different ( $p<0.05$ ) when compared with LPS-activated macrophages

### 3.6 Effects of *P. frutescens* leaves extracts on NO production

The effects of *P. frutescens* leaves extracts on NO production in LPS-stimulated RAW 264.7 cells are shown in Table 3. RAW 264.7 cells were treated with various concentrations of *P. frutescens* leaves extracts (0.015625, 0.03125, 0.0625, 0.125, 0.25 and 0.5  $\mu$ g/mL) and LPS (1  $\mu$ g/mL) for 24 h. The *P. frutescens* leaves extracts were evaluated for the inhibition of NO production in the LPS-stimulated RAW 264.7 cell. The nitrite accumulation in the cells increased due to the LPS treatment. The cells were simultaneously treated with 1  $\mu$ g/ml LPS and different concentrations of the extracts. The RAW 264.7 cells were activated by LPS and NO production was measured as nitrite concentration. The extracts at different concentrations significantly inhibited ( $p<0.05$ ) the nitrite accumulation in LPS-stimulated RAW 264.7 cells in the concentration-dependent manner.

The results showed that the 040P extract at the concentrations 0.125 and 0.25 mg/ml had significant ( $p<0.05$ ) efficacy in inhibiting activity of NO production in the LPS-stimulated RAW 264.7 cells which were  $20.25\pm9.36$  and  $75.57\pm10.72\%$ , respectively; while the 112P and 115P are significantly inhibited ( $p<0.05$ ) NO production in the LPS-stimulated RAW 264.7 cells which were  $43.31\pm8.84$  and  $33.97\pm2.51\%$  (at the concentration 0.25 mg/ml) and  $93.54\pm4.02$  and  $74.29\pm10.19\%$  (at the concentration 0.50 mg/ml), respectively.

The 040E and 112E extracts at the concentrations 0.0625, 0.125, 0.25 and 0.5 mg/ml

showed significant ( $p<0.05$ ) efficacy in inhibiting activity of NO production in the LPS-stimulated RAW 264.7 cells ( $78.45\pm15.12$ - $98.37\pm2.82$ ); while the 115E significantly inhibited ( $p<0.05$ ) NO production in the LPS-stimulated RAW 264.7 at the concentration 0.015625-0.125 mg/ml ( $52.49\pm8.90$ - $88.88\pm12.88$ ) when compared to the cells which were treated with LPS.

The result of MTT cell viability assay revealed that the inhibitory effect of the petroleum ether and ethanol extract of *P. frutescens* leaves decreased NO production and did not damage cell (viability  $>95\%$ ). The observation of activities is does-dependent concentrations.

The anti-inflammatory properties of the extracts were evaluated on the basis of their inhibition of NO and LPS activated macrophages. Nitric oxide is an important intracellular and intercellular regulators of multiple biological functions, including macrophage-mediated cytotoxicity, neurotransmission and smooth muscle relaxation. Overexpression of NO has been associated with oxidative stress and the pathophysiology of various diseases such as autoimmune disease and chronic inflammation [25]. The cell toxicity of the extracts was determined by MTT assay. The results indicated that most of the extracts showed significant cell viability at a concentration of 0.25 mg/ml ( $> 95\%$ ), except 115E. Therefore, the extracts of *P. frutescens* leaves exhibited low toxicity. All of the extracts also inhibited macrophage NO production. The results also suggested that the high value of total

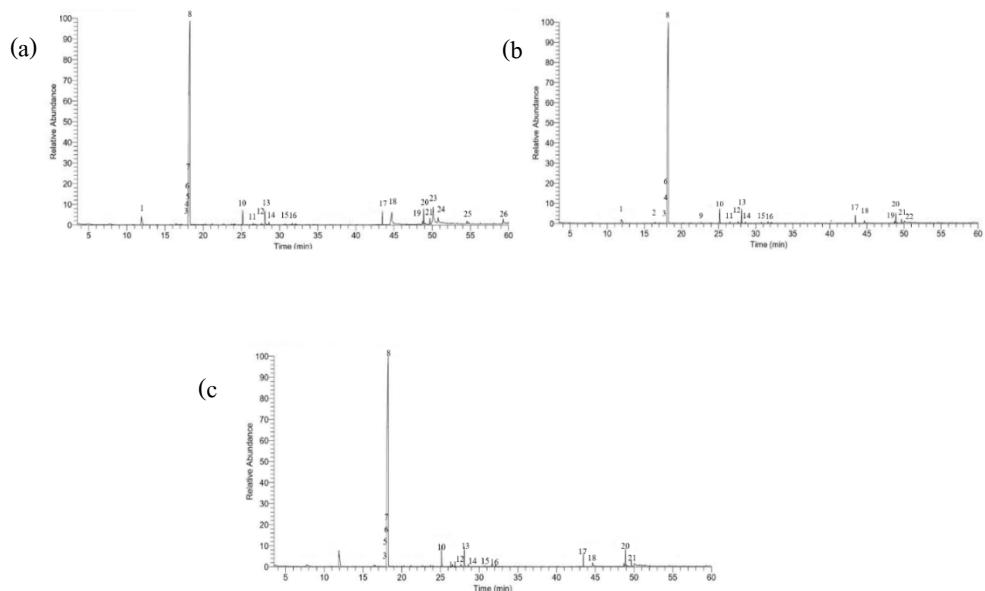
phenolic led to statistically high anti-inflammatory potential.

The percentage of inhibition NO production of *P. frutescens* leaves extracts was significantly increased in a dose-dependent manner ( $p<0.05$ ). However, excessive NO production can result in the development of inflammatory diseases such as rheumatoid arthritis and autoimmune disorders. Thus, inhibition of NO production is a major target for anti-inflammatory agent development [18]. From the results of bioactivity test shown in Table 1, 040E offered the highest activity. In addition, from anti-inflammatory activity results in Table 3, 040E and 112E delivered the great amount of activity. Therefore, we selected 040E to be added into the best cream base (040E cream).

### 3.7 Composition of volatile compounds in *P. frutescens* leaves

The volatile components of the *P. frutescens* leaves were analyzed by GC-MS. The extracts by hydro-distillation were identified twenty-six volatile compounds. The chromatograms of the identified volatile compounds are shown in Figure 1. The dominant volatile component was 2-hexanoylfuran, which found in all samples (040H; average content 53.96%, 112H; average content 41.56% and 115H; average content 36.66%), followed by 1-pentanone (H112; average content 17.80%, H115 average content 15.08%, H040; average content 7.73%).

The data showed that linalool, caryophyllene, humulene, germacrene D,  $\alpha$ -bergamotene,  $\alpha$ -(Z,E)-farnesene, trans-nerolidol, caryophellene oxide, hexadecanoic acid, methyl ester, n-hexadecanoic acid, methyl 9-cis, 11-trans-octadecadienoate, trans-13-octadecenoic acid, methyl ester and methyl stearate were found in three sample. Moreover, we also observed 3-allylguaiaol and oleic acid in 112H whereas cis-vaccenic acid, octadecanoic acid, clycidyl palmitate, and glycidyl oleate were found in 040H as shown in Figure 1. The different volatile components between *P. frutescens* leaves sample could be considered as the different varieties of frutescens species that could be analyzed by PCR technique for variety identification. Furthermore, it has been reported that linalool and the fatty acid; oleic acid, cis-vaccenic acid, n-hexadecanoic acid are also known to be anti-oxidant and anti-inflammatory activity [29-32]. Since *P. frutescens* contains the essential oil, there are aroma compound, and therefore it can be used as flavoring agents in food and fragrance ingredients for cosmetic products. The researches on the volatile essential oil components of *P. frutescens* indicate that it is an especially good source of perilla aldehyde, perilla ketone,  $\beta$ -caryophyllene, benzaldehyde, and limonene which contain antioxidant, anti-inflammatory, antibacterial, antifungal, anti-allergic, anticancer, antidepressant, and antiproliferative activities [33].



**Figure 1** GC-MS chromatogram of essential oil components from the leaves of *P. frutescens*.

(a) GC-MS chromatogram of the 040H. (b) GC-MS chromatogram of the 112H. (c) GC-MS chromatogram of the 115H. 1. Linalool, 2. Unknown, 3. 1-Pentanone, 4. Unknown, 5. Unknown, 6. Unknown, 7. Unknown, 8. 2-Hexanoylfuran, 9. 3-Allylguaiacol, 10. Caryophyllene, 11. Humulene, 12. Germacrene D, 13.  $\alpha$ -Bergamotene, 14.  $\alpha$ -(Z,E)-Farnesene, 15. trans-Nerolidol, 16. Caryophellene oxide, 17. Hexadecanoic acid, methyl ester, 18. n-Hexadecanoic acid, 19. Methyl 9-cis, 11-trans-octadecadienoate, 20. trans-13-Octadecenoic acid, methyl ester, 21. Methyl stearate, 22. Oleic acid, 23. cis-Vaccenic acid, 24. Octadecanoic acid, 25. Clycidyl palmitate, 26. Glycidyl oleate

### 3.8 Stability and skin irritation test

The stability of 040E cream was stable at various storage conditions, the results showed that its physical properties, including pH and appearance in all conditions did not change after storage ( $p < 0.05$ ) (Data not showed). After that the dermal skin irritation test was investigated. The test substances presented that the deionized water, *P. frutescens* leaves extract, cream base and cream containing *P. frutescens* leaves extract (040E

cream) were non-irritating with low Primary Dermal Irritation Index value ( $PDII < 0.5$ ); whereas 1 % w/v SLS, which was used as a positive control, was found to be slightly-irritating ( $PDII$  range from 0.5 to 2.0) (Table 4). The 040E cream showed  $PDII$  value lower than cream base, this can be a result of anti-inflammatory activity obtained from the *P. frutescens* leaves extract which incorporated in the 040E cream. This result suggested that the test creams were safe to apply to skin.

**Table 4** The Primary Dermal Irritation Index (PDII) and skin irritation reaction observed in thirty healthy volunteers.

Test substances	PDII value	Classification of skin Irritation
1 % w/v SLS (Positive control)	0.86	Slightly-irritating
Deionized water (Negative control)	0.00	Non-irritating
<i>P. frutescens</i> leaves extract	0.07	Non-irritating
Cream base	0.18	Non-irritating
040E cream	0.11	Non-irritating

#### 4. Conclusions

*P. frutescens* leaves showed a potential as source of natural antioxidants, free radical scavenging compounds, serving as anti-inflammatory agent, and presented many essential oils. It might be a good plant-based pharmaceutical product for several diseases such as age-related disorders, cancer, neurodegenerative diseases and inflammation caused by free radicals. Besides, their information can support the use as an edible plant. Furthermore, essential oils as non-polar molecules can be incorporated in cosmetic products since they can be easily penetrate the skin and their pleasant odor.

Anti-inflammatory activity from *P. frutescens* has a potential to be used safely in the cosmetics products for irritant reduction which is suitable for sensitive skin.

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