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Contributed Paper

Anti-inflammatory, Antioxidant Activities and Safety of *Coffea arabica* Leaf Extract for Alternative Cosmetic Ingredient

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ABSTRACT

Coffee (*Coffea arabica*) or Arabica is grown as an economic cash crop in the northern highlands of Thailand. Utilization of coffee leaves has historically been overshadowed by coffee bean products. Phytochemical constituents, antioxidant and anti-inflammatory activities as well as irritation of Thai arabica coffee leaf extract were evaluated. The leaf extract demonstrated potential antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging, and lipid peroxidation inhibition assays, with high phenolic content and flavonoid content. Anti-inflammatory potential was observed in dose-dependent manner by reducing interleukin-6 (IL-6) secretion from LPS-stimulated macrophages. Irritation on the chorioallantoic membrane of hen's eggs were not observed. Therefore, coffee leaf extract has potential as the new alternative ingredient for cosmetics or cosmeceutical products. Moreover, this research has benefit for adding the value to the coffee leaves in off-season for coffee beans.

Keywords: *Coffea arabica*, leaf extract, anti-inflammatory activity, antioxidant, irritation test

1. INTRODUCTION

Aging is an unavoidable process that occurs due to cellular degradation over time. Free radicals are now recognized as one of the primary causes of aging through oxidative stress. Many factors generate free radicals including pollution, stress, UV radiation and environmental changes. Free radicals can induce several chronic diseases such as cancer, coronary heart disease, neuropathy, lung disease and rheumatoid arthritis [1]. Furthermore, free radicals affect aesthetic appearance by generating wrinkles, dark spots, roughness, dryness and loss of skin elasticity. Skin aging can be divided into two types as

intrinsic aging and extrinsic aging. Intrinsic aging is resulted from metabolic processes and hormones within the human body, while extrinsic aging is caused by external factors, especially UV radiation. Imbalance between free radical production and cellular antioxidant mechanisms within the cells lead to the oxidative stress. Therefore, antioxidants are of interest to prevent the occurrence of free radicals as well as scavenge them. Antioxidant compounds can be both synthetic and natural. Nowadays, natural antioxidants have highly attracted interest due to their efficacy, safety and cost. Some of the best antioxidant components are phenolic compounds which are abundant in many types of herbal plants, vegetables and fruits [2].

Inflammation is a complex immune response related to host defense mechanisms against infection, injury, and also many chronic diseases such as arthritis, cancer, atherosclerosis, and asthma. Cytokines, as small protein molecules, play a crucial role in both innate and adaptive immune responses. They are produced by several cell types, especially macrophages and helper T-cells through using a nuclear factor (NF-kB) signaling pathway, which is able to communicate with other cells as well as regulate the expression of inflammation [3, 4]. Interleukin-6 (IL-6) is a pleiotropic cytokine contributed to the transition between acute and chronic inflammations as well as pain reaction [3-5]. Thus, IL-6 is recognized as a target for inflammatory treatment [5]. Therefore, compounds exerting the inhibitory effect on IL-6 secretion might be promising agents for treating inflammation.

Coffee (*Coffea arabica*) or Arabica is one the most popular beans used as a raw material of coffee beverages, which are ubiquitously consumed worldwide, especially Asia. Coffee is grown as an economic crop throughout the northern Thailand including Chiang Mai, Chiang Rai, Lampang and Mae Hong Son. The coffee tree belongs to the Rubiaceae family. It is a small tree (2 to 8 m) with sweet-scented white flowers and evergreen shiny leaves. Anti-inflammatory and antioxidant activities of green and roasted coffee beans have been previously investigated [6-8]. Aside from coffee beans, extract of young coffee leaves consisted of higher caffeine, theobromine and caffeoylquinic acid than fully developed leaves [9, 10]. These components are good antioxidant compounds, especially 3-, 4-, and 5-caffeoylquinic acid as the main chlorogenic acids found in nature. In addition, Chen (2018) reported whether the aqueous extract of coffee leaves (Arabica) exerted antioxidant and anti-inflammatory effects attributed to its phenolic compounds [11]. However, the biological activity of an ethanolic extract of coffee leaf has not been reported yet. Therefore, the aims of this study were to analyze phytochemical constituents and study antioxidant, anti-inflammatory activities along with irritation profile of the ethanolic extract of Thai arabica coffee leaf that the data will be further used to develop cosmetic, cosmeceutical or health products. In addition, it could improve the value of coffee leaves in off season for coffee beans.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,22-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), linoleic acid, and 2,22-Azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Fluka (Buchs, Switzerland). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Trolox, gallic acid, rutin, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl

tetrazolium bromide (MTT), and caffeine were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modied Eagle medium (DMEM), Penicillin-Streptomycin, L-glutamine, and trypan blue were purchased from Invitrogen™ (Grand Island, NY, USA). Ethanol, methanol, and acetonitrile were purchased from Labscan Asia Co., Ltd. (Thailand). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (Darmstadt, Germany). Sodium carbonate, aluminium chloride and sodium nitrite were purchased from United Chemical & Trading CO., Ltd. (Thailand).

2.2 Plant Material

Leaves of *C. arabica* were collected from Chiang Mai, Thailand, then washed and dried in a hot air oven at 40°C for 24 h. The dry leaves were ground by blender to reduce the size and stored in plastic jar at room temperature before extraction.

2.3 Extraction of *C. arabica* Leaves

C. arabica leaves were extracted with 95% (%v/v) ethanol by maceration for 72 h. The extract solution was filtered using Whatman filter paper No.1 and the marc was re-extracted with 95% (%v/v) ethanol twice. The filtrate was evaporated by a rotary evaporator. The *C. arabica* leaf extract (LE) was kept in an amber bottle at 4°C prior to analysis.

2.4 Chemical Constituent of *C. arabica* Leaf Extract

The chemical composition of the leaf extract was analyzed by HPLC model 1100 (Agilent Technologies, USA). A Mightysil® C18 column (5 μm, 250 × 4.6 mm ID) was used as stationary phase. The mobile phase was isocratic elution that composed of acetonitrile mixed with 0.5% acetic acid and 1% acetic acid in deionized water (85:15 v/v, pH 3) with flow rate 1 mL/min. The sample was prepared by dissolving the extract (0.005 mg/mL) in methanol and filtration through a 0.45 μm nylon syringe filter. Then, the sample (10 μL) was injected into the HPLC column which was maintained at 25°C. The chromatogram was measured by UV detector at 280 nm using caffeine, chlorogenic acid and ferulic acid as standards.

2.5 Determination of Total Phenolic Content

The *C. arabica* extract was analyzed for total phenolic content by Folin-Ciocalteu assay [12]. The extract was dissolved in ethanol (1 mg/mL). Then, the sample (500 μL) was transferred into a test tube, mixed with 2 mL of 10% v/v Folin-Ciocalteu reagent in deionized water, and 4 mL of 7.5% w/v sodium carbonate was added. The solution was mixed and incubated for 30 min in the dark. Absorbance was measured at 765 nm using a spectrophotometer. Concentration of total phenolic content in the extract was calculated as gallic acid equivalent (GAE) in milligram gallic acid/gram of dry sample.

2.6 Determination of Total Flavonoid Content

Total flavonoid content was determined following the method of Samatha *et al.* with some modifications [13]. An aliquot (1 mL) of the extract, distilled water (10 mL) and 5% sodium nitrite (0.3 mL) was mixed and incubated for 5 min. Then, 10% aluminium chloride was added in the mixture and incubated in the dark for 30 min. Absorbance was measured at 765 nm using a spectrophotometer. Concentration of total flavonoid content was calculated as rutin equivalent (RE) in milligram rutin/gram of dry sample.

2.7 Determination of Antioxidant Activity

2.7.1 DPPH radical scavenging assay

Scavenging activity of the extract on DPPH radicals was determined using the modified method of Brem *et al.* [14]. Different concentrations of the extract (2, 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL) were dissolved in ethanol. An aliquot of sample (20 μL) was mixed with 180 μL of 167 μM DPPH solution and kept in the dark for 30 min. Absorbance was measured spectrophotometrically at 520 nm with a microplate reader. Trolox and gallic acid were used as standards. Percentage inhibition was calculated using the following equation:

$$\%\text{inhibition} = [(Ac - As)/Ac] \times 100\%$$

Where Ac is the absorbance of control and As is the absorbance of sample. The half maximal inhibitory concentration (IC_{50}) was calculated from the curve between the percentage of inhibition and the concentration of extract.

2.7.2 ABTS radical scavenging assay

ABTS stock solution was prepared by mixing 7 mM ABTS solution with 140 mM potassium persulfate solution and kept in the dark at room temperature for 16 h before use [15]. The solution was diluted with deionized water to obtain an absorbance of 0.9 ± 0.1 at 734 nm. The extract was dissolved in ethanol at different concentrations. An aliquot of sample (10 μL) was added to 1 mL of ABTS solution. The mixture was measured for absorbance at 734 nm after 6 min. Percentage of inhibition and IC_{50} were calculated from the same equation as used for DPPH-radical scavenging assay.

2.7.3 Lipid peroxidation inhibition assay

Linoleic acid was used for the lipid peroxidation inhibition assay following the method of Olszewska with modifications [16]. The extract was diluted with ethanol before use. An aliquot of sample (200 μL) was mixed with 800 μL of phosphate buffer (pH 7.0), 200 μL of ethanol, 400 μL of deionized water, and 400 μL of 2.5% linoleic acid in a test tube. AAPH solution (80 μL) was added in the mixture and incubated in the dark at 37 °C for 24 h to generate lipid peroxidation. The result was tested by the ferric thiocyanate method. The mixture was mixed with 20 mM ferrous chloride in 0.1 mL of 3.5% hydrochloric acid, 0.1 mL of 10% ammonium thiocyanate solution, and 9.7 mL of 75% methanol for 5 min. Absorbance was measured at 500 nm using a spectrophotometer and the percentage of inhibition was calculated from the same equation used for DPPH-radical scavenging assay. The IC_{50} value was determined from the regression curve of the concentration.

2.8 Anti-Inflammation Activity

2.8.1 Determination of IL-6 secretion from LPS-stimulated RAW 264.7 cells

Lipopolysaccharide (LPS)-stimulated RAW 264.7 cells (American Type Culture Collection, ATCC-TIB-71) were used to investigate the inhibitory effects of the coffee extract or standard (caffeine) on IL-6 secretion following the modified method of Mueller *et al.* [17]. Briefly, the cells were maintained in DMEM supplemented with heated-inactivated 10% fetal bovine serum (FBS), 4 mmol/L L-glutamine, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C in a humidified atmosphere with 5% CO_2. The cells were then seeded in 24-well plate to give a final cell density of 2×10^6 cells/well for 24 h, and subsequently incubated with or without the extracts or standard for 2 h before adding LPS to give a final concentration of 1 μg/mL. The cells

were incubated for 24 h and the supernatants were collected to determine the percentage of IL-6 secretion using ELISA assay kit (R&D Systems, Minneapolis, MN, USA). All ELISA experiments were performed at room temperature. Absorbance was measured using a Genios Pro microplate reader (Tecan, Crailsheim, Germany) at UV-wavelength of 450 nm and reference wavelength of 570 nm. Cells incubated with only LPS were used as positive control while cells without LPS were negative control.

Along with the ELISA assay, the cells remaining in 24-well plates were incubated with 50 μL of MTT dye (5 mg/mL) for 2 h. The medium was then removed, and lysis buffer was added. Percentage cell viability was measured using a Genios Pro microplate reader at UV-wavelength of 450 nm and reference wavelength of 570 nm.

Normalized IL-6 secretion was adjusted by percentage cell viability following this equation;

$$\text{Normalized IL-6 secretion} = [\%\ \text{IL-6 secretion} / \%\ \text{cell viability}] \times 100.$$

Positive control was defined as 100% cytokine secretion. The IL-6 produced from the cell was recorded as a percentage of this value. All experiments were performed in triplicate.

2.9 Irritation Test Determined by Hen's Egg Test Chorioallantoic Membrane (HET-CAM)

Irritation score was tested by using the hen's egg test chorioallantoic membrane (HET-CAM) following the method used by Chaiyana *et al.* [18]. Hen eggs were obtained after fertilization from the Faculty of Agriculture, Chiang Mai University. All eggs were incubated for 7 days in the hatching chamber with 37.5 ± 0.5°C, humidity 55 ± 7%. To prepare the CAM, the eggshell was opened with an electric drill and the white egg membrane was removed. The extract was dissolved in DI water and added to the CAM. Appearance of the membrane and its blood vessel was evaluated as hemorrhage, lysis, and coagulation 5 min after adding the sample. Times of rst occurrence of the three above-mentioned endpoints were recorded. Irritation index (IS) was calculated using the following equation:

$$IS = [(301-t(h))/300\times5] + [(301-t(l))/300\times7] + [(301-t(c))/300\times9]$$

Where t(h) is the time(s) when the rst vascular hemorrhage was detected, t(l) is the time(s) when rst vascular lysis was detected, and t(c) is the time (s) when the rst vascular coagulation was detected. Irritation score (IS) was then evaluated as follows: 0.0-0.9, no irritation; 1.0-4.9, mild irritation; 5.0-8.9, moderate irritation; and 9-21, severe irritation [19]. The CAM was captured under the microscope by Lumix digital camera (Panasonic, Beijing, China) after the experiment.

2.10 Statistical Analysis

All data were presented as a mean ± standard deviation (SD). Statistical analysis used the SPSS statistic version 17.0 program. Individual differences were evaluated by t-test or one-way ANOVA. In all cases, $p < 0.05$ indicated statistical signicance.

3. Results and Discussion

3.1 Percentage Yield and Chemical Constituents of The *C. arabica* Leaf Extract

The arabica leaf extract (LE) was obtained by maceration with ethanol. The extract was a sticky semisolid with dark

green color and gave percentage yield as 6.25% ± 1.0 with pH 5. The HPLC chromatogram of the LE (0.005 mg/mL) as shown in Figure 1 presented a major peak at the retention times of 7.921, 8.760 and 13.420 min, which were similar to the retention time with caffeine, ferulic acid and chlorogenic acid standards. The amount of caffeine, ferulic acid and chlorogenic acid in the LE was 22.76, 4.47 and 7.92 μg/ 1 mg of extract, respectively. Ashihara reported that the amount of caffeine in leaves of arabica coffee was higher than those of theobromine and xanthine [10]. Caffeoylquinic acid or chlorogenic acid, mangiferin, and hydroxycinnamic acid esters were also found in arabica coffee leaves [9, 11, 20]. These compounds could be extracted by polar solvent. However, phenolic compounds were detected at low levels in arabica coffee leaves.

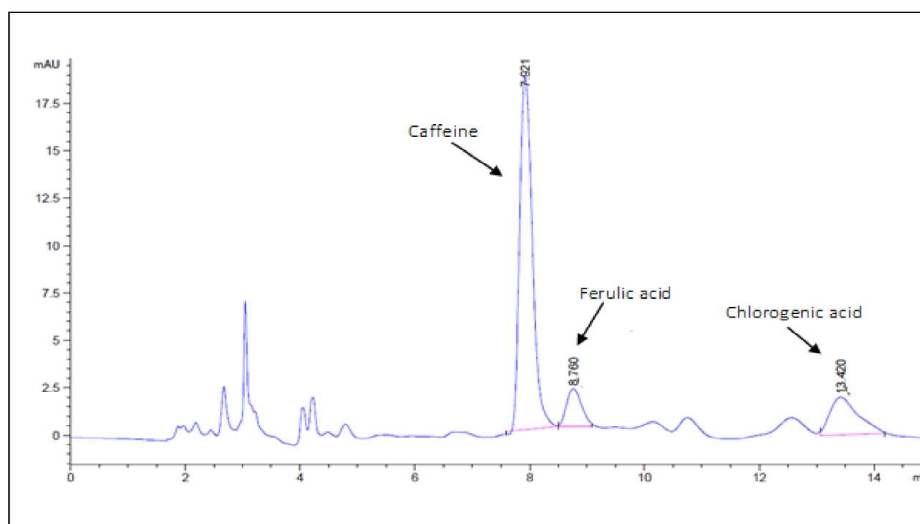

Figure 1. Chromatograms of the LE using HPLC at wavelength of 280 nm.

3.2 Determination of Total Phenolic and Total Flavonoid Contents

Phenolic compounds, which are commonly found in plants, possess potent antioxidant activity. Flavonoids are in a ubiquitous group in the phenolic compounds, that also possess high antioxidant activity. Therefore, the extract containing high total phenolic and flavonoid contents is regarded as good antioxidant agents. Total phenolic and flavonoid contents of the arabica leaf extract are presented in Table 1. Total phenolic content of the LE was 319.2 mg gallic acid/g of extract with total flavonoid content as 350.0 mg rutin/g of extract. Polar or semipolar solvents including ethanol, methanol and their aqueous mixtures are commonly used to extract polyphenols from plant material. Besides, solubility profiles of the phenolic compounds in the solvent can be used for determining the extraction process. The amounts of polyphenols and flavonoids were influenced by the variations of plant materials, solvent types and extraction time [21]. Campa *et al.* found that coffee leaves contained phenolic compounds, mangiferin, and hydroxycinnamic acid (chlorogenic acid, caffeic acid, ferulic acid) that related to the total phenolic and flavonoid contents of the LE, found in our study including the data from HPLC chromatogram [20].

Table 1. Total phenolic and flavonoid contents, along with antioxidant activities of the arabica leaf extract (LE) and standards by various assays (n = 3).

Sample	Total phenolic content (mg gallic acid/g extract)	Total flavanoid content (mg rutin/g extract)	DPPH assay IC_{50} (mg/mL)	ABTS assay IC_{50} (mg/mL)	Lipid peroxidation assay IC_{50} (mg/mL)
LE	319.2 ± 2.8	350.0 ± 0.1	0.070 ± 0.0	0.014 ± 0.0	2.518 ± 0.0
Trolex	-	-	0.005 ± 0.2	8.64×10^{-4} ± 0.0	0.047 ± 0.0
Gallic acid	-	-	0.002 ± 0.3	5.99×10^{-4} ± 0.0	0.124 ± 0.0
Quercetin	-	-	0.006 ± 0.2	5.38×10^{-4} ± 0.0	0.083 ± 0.0

3.3 Antioxidant Activity of The *C. arabica* Leaf Extract

Antioxidant agents, especially derived from plant, contain a lot of biological components, which involve in many different antioxidant mechanisms. Therefore, various antioxidant assays were performed. The antioxidant activity relates to the amounts of polyphenols and other active components in the extract. Table 1 presents the antioxidant activity of the LE, determined by DPPH, ABTS, and lipid peroxidation inhibition assays. The DPPH scavenging effect of LE was comparable to that of green coffee bean extract (IC_{50} 0.05 mg/mL) and definitely better than that of roasted coffee bean extracts (IC_{50} 0.18 mg/mL) as mentioned in our previous report [22]. Besides, ABTS-radical scavenging assay was performed to confirm the radical scavenging activity. It was shown that the LE also exhibited good ABTS scavenging activity, which was better than green and roasted coffee bean extracts (IC_{50} 0.016 and 0.024 mg/mL) [22]. Interestingly, the IC_{50} value, reported by ABTS assay, was lower than the value from DPPH assay. This is due to the fact that the ABTS radical shows higher polarity than DPPH radical. Thus, ABTS assay is appropriate for the polar compounds. From the results, scavenging activity of the extract was in correspondence with the total phenolic content. In addition, linoleic acid peroxidation assay was carried out to determine the protective effect of the extract on lipid peroxidation. Linoleic acid served as an unsaturated fatty acid, which was oxidized by AAPH radical. The IC_{50} value of the LE on linoleic acid peroxidation was higher than the IC_{50} values from DPPH and ABTS assays (Table 1). The explanation might be due to the polarity of phenolic compounds within the extract, which were soluble in polar solvents. Therefore, the LE was not compatible with the lipid peroxidation inhibition method.

3.4 Anti-inflammation Activity of The *C. arabica* Leaf Extract

Anti-inflammatory effects of the LE and its major constituents were evaluated by IL-6 secretion levels of RAW 264.7 cells after LPS treatment. The results demonstrated that all concentrations of the LE did not show cytotoxicity, since viability of RAW 264.7 cells after exposure to the extract presented non-significant difference from the control cells. Our study firstly illustrated the anti-inflammatory effect of the ethanolic extract of the arabica coffee leaf. The LE at concentrations of 0.1 and 0.075 mg/mL along with caffeine at concentrations of 0.1, 0.075, and 0.050 mg/mL presented inhibitory effect on IL-6 secretion from

LPS-induced RAW 264.7 cells (Figure 2). The amount of IL-6 secretion after treatment with the LE at high concentrations were significantly reduced compared to cells treated with LPS alone. The inhibitory effect on IL-6 production of LE was dose-dependent. Caffeine, a main component of the extract, was widely reported for its anti-inflammatory effect, which involved in several pathways [23, 24]. Hwang *et al.* (2016) denoted that caffeine reduced the expression of pro-inflammatory genes including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-3, IL-6, and IL-12, which was in a good agreement with our results [24]. Caffeine exerts anti-inflammatory effect due to its ability of regulating NF-kB pathway, which plays a crucial role in pro-inflammatory cytokine production [25]. IL-6 is generally produced by keratinocytes and the secretion is enhanced by UV radiation [26]. Pro-inflammatory cytokines play an important role in sunburn, skin damage, and premature aging together with the increase of melanomas risk and other types of skin cancer. In addition, chronic elevated levels of IL-6 in aged skin leads to the downregulation of collagen synthesis and the upregulation of matrix metalloproteinases (MMPs), enzymes degrading collagen. As a result, the LE might be a potential anti-aging agent due to its notable anti-inflammatory and antioxidant properties.

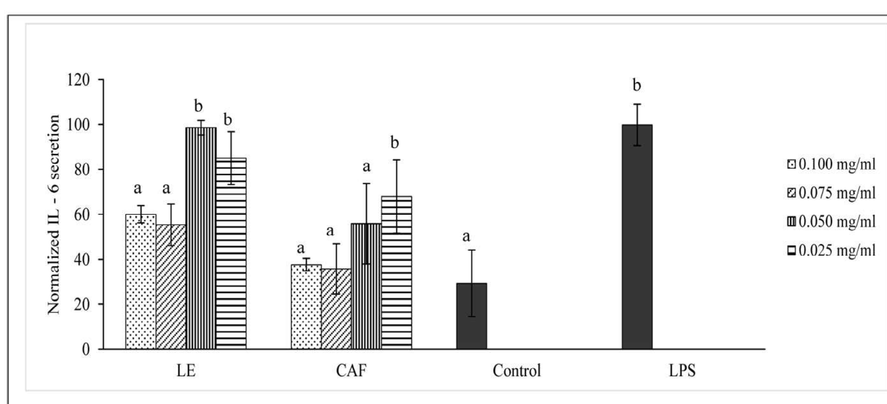

Figure 2. Normalized IL-6 secretion from LPS-stimulated RAW 264.7 cells incubated with the extracts in various concentrations; ( ) 0.100 mg/mL, ( ) 0.075 mg/mL, ( ) 0.050 mg/mL, ( ) 0.025 mg/mL. Different superscript letters (a and b) indicate significant difference ($p < 0.05$) between groups based on one-way ANOVA with multiple comparison using Tukey's range test.

3.5 Irritation Test Determined by Hen's Egg Chorioallantoic Membrane (HET-CAM)

The HET-CAM assay is widely used to evaluate the irritation potential of substances, plant extracts, cosmetic products, and drug delivery systems [27-30]. Three reactions were determined including hemorrhage, lysis and coagulation of the chorioallantoic membrane of the fertilized chicken egg after treatment with test samples. Irritation results are shown in Table 2. The LE showed no irritation similar to 0.9% sodium chloride. On the contrary, severe irritation was observed in 1% w/v sodium lauryl sulfate (SLS) which is widely used in cosmetic products and causes skin irritation. Blood vessel appearances before and after treatment with the samples

are shown in Figure 3. SLS produced vascular hemorrhage, vascular lysis and vascular coagulation, whereas the LE did not show any adverse vascular effects, eye irritation or skin irritation. Further studies are required to determine its potential as an alternative ingredient in cosmetic or cosmeceutical products.

Table 2. Irritation index after treatment with the arabica leaf extract (LE), 0.9% sodium chloride and 1% sodium lauryl sulfate.

Sample	Irritation index (IS) score	Irritation level
LE	0	No irritation
Negative control (0.9% w/v NaCl)	0	No irritation
Positive control (1% w/v SLS)	11.38 ± 0.03	Severe irritation

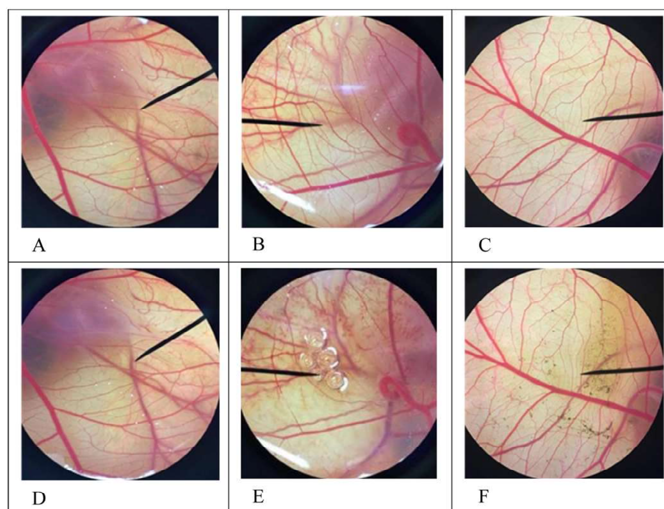

Figure 3. Appearance of blood vessels before and after treatment with 0.9% sodium chloride (A and D), 1% sodium lauryl sulfate (B and E) and the arabica leaf extract (C and F).

4. Conclusions

Antioxidant activity, IL-6 secretion in LPS-induced macrophages, and irritation of the arabica leaf extract were determined. It possessed potential antioxidant activity related to its phenolic and flavonoid contents. It also showed anti-inflammatory potential with dose-dependent IL-6 secretion reduction *in vitro*. It showed no cytotoxicity in macrophages and did not irritate the chorioallantoic membrane of hen's eggs. Therefore, investigation of the antioxidant, anti-inflammatory activities and safety of Arabica coffee leaf extract is of interest to add value to the coffee leaves during the off-season for coffee beans. Research data might be financially beneficial to coffee farmers, especially during periods of coffee bean price fluctuation, while providing consumers with a new active ingredient for cosmetic, cosmeceutical and health products.

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