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Contributed Paper

Antioxidant, Anti-tyrosinase, Anti-aging Potentials and Safety of Arabica Coffee Cherry Extract

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Abstract

The coffee cherry (*Coffea Arabica*) is a by-product from the coffee production process that is widely used in agriculture; however, there are few reports regarding the application of coffee by-products in cosmetics. Therefore, this study aimed to investigate the antioxidant, anti-tyrosinase and anti-aging activities as well as the toxicity of coffee cherry extracts. The extracts of the coffee cherry fruits were obtained by fractionated extraction using hexane, ethyl acetate, and ethanol, respectively. Anti-aging bioactivities were investigated. Results showed that the ethyl acetate extract was shown to contain the highest total phenolic content (301.96 ± 4.05 mg GAE/g extract). It possessed good antioxidant activity according to the DPPH (IC_{50} value as 35.65 ± 1.35 µg/mL) and FRAP assays (FRAP value as 100.52 ± 0.08 mM $FeSO_4$/g) that the results were higher than caffeine standard. The ethyl acetate extract also showed the highest anti-tyrosinase activity (IC_{50} values as 0.23 ± 0.01 mg/mL and 0.98 ± 0.02 mg/mL when used L-tyrosine and L-dopa as substrates) when compared with other extracts. However, it showed lower anti-tyrosinase activity than kojic acid and vitamin c. It also demonstrated the highest inhibition against matrix metallopeptidase-2 and hyaluronidase (% inhibition as 25.44 ± 2.1 and 92.95 ± 0.61) when compared with other extracts. Moreover, it was found to be non-toxic to NIH-3T3 cells according to the MTT assay with concentrations 8-800 µg/mL, and an irritation effect was not observed in HET-CAM assay (irritation score was 0.0, at the concentration of 10 mg/mL). Therefore, this coffee cherry extract could be an attractive natural extract for further development of whitening or anti-aging products. Moreover, this can be a way to develop the value of arabica coffee waste into a value product that can be applied in the cosmetics industry.

Keywords: *Coffea arabica*, coffee cherry, antioxidant, anti-aging, safety testing

1. INTRODUCTION

Coffee (*Coffea Arabica* or *C. arabica*) is among the most popular beverages and widely consumed throughout the world, including Asia. The coffee tree belongs to the Rubiaceae family [1]. Nowadays, production of arabica has dramatically increased in northern Thailand. The higher coffee consumption generates the increased by-products of coffee. The by-products from the coffee production process and the coffee shop brewing business are coffee cherries and coffee grounds, which cause significant environmental problems. The coffee cherry or coffee fruit is a smooth outer skin or pericarp, usually green in unripe fruits but red-violet or deep red when ripe. It must be removed during the process to separate the beans. The coffee cherries have no commercial importance. However, they can be used as fertilizer, compost and substrate for growing exotic mushrooms such as Shiitake, Linch, Pleurotus or Oyster [2]. They are also used as dairy animal feed due to containing protein, carbohydrates, minerals and fiber [2,3]. In spite of the fact that using coffee by-products is limited due to their high caffeine and tannin contents, coffee husks have been used as the substrate for the production of aroma compounds for the food industry through use of yeast and fungi. Thus, it is an alternative choice for naturally produced food and flavor compounds [4]. Moreover, coffee pulp and husks are also used for biogas production in anaerobic digestion [4,5]. In addition, the dry coffee pulp can also be used to produce briquettes and pellets [6]. For health science, it has been reported that coffee cherries have good antioxidant activity, which may be due to the composition of caffeine and phenolic compounds such as chlorogenic acid (5-caffeoylquinic acid), epicatechin, catechin, rutin, protocatechuic acid and ferulic acid [4]. Until now, there has been little research conducted regarding the application of coffee by-products in cosmetics. Additionally, people currently tend to be increasingly concerned about health and beauty due to environmental issues, pollutants, stress, and processed and fast food. Many factors cause the activation of free radical formation in our bodies. These free radicals can result in numerous diseases such as cancer, coronary heart disease, neuropathy, lung disease, and rheumatoid arthritis as well as skin problems [7]. In addition, there are various factors that lead to the aging of the skin due to changes in the dermal extracellular matrix involving collagen, elastin, hyaluronic acid, etc. Therefore, the researcher has developed the idea to change the waste products into value products that can be applied in the cosmetics industry. The aims of this research were focused on the extraction, investigation of biological activities and toxicity of coffee cherry extracts. These will be employed as the process to examine the value of arabica coffee waste.

2. MATERIALS AND METHODS

2.1 Chemical Materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, and 2,22-Azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Fluka (Buchs, Switzerland). Folin-Ciocalteu reagent and bromophenol blue were purchased from Merck (Darmstadt, Germany). Hexane, ethanol, ethyl acetate, hydrochloric acid (HCl), dimethyl sulfoxide (DMSO), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), calcium chloride ($CaCl_2$), and methanol were purchased from Labscan Asia Co., Ltd (Thailand). 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), chlorogenic acid, caffeic acid, caffeine, gallic acid, ammonium thiocyanate (NH_4SCN), hyaluronidase from bovine testis, ferrous sulfate ($FeSO_4$), ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$), 2,4,6 tripyridyl-s-triazine (TPTZ), mushroom tyrosinase, L-dopa, L-tyrosine, Triton X-100, glycerol, hyaluronic acid, alcian blue 8GX, and gelatin were purchased from Sigma-Aldrich

(St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), coomassie blue and protein markers were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Acetic acid and Tris base were purchased from Fisher Chem Alert (Fair Lawn, NJ, USA).

2.2 Plant Material

Coffee cherry fruits of *Coffea arabica* were collected during the period of November to December 2017. It was grown in Chiang Mai province, Thailand. Only the ripe coffee cherry fruits or the pulp of coffee cherries were washed and dried in hot air oven at 50 °C for 24 h. Then, the dried coffee cherry fruits were grinded into fine powder by blender (600 W, Viva Collection Blender, Phillip) at room temperature before the extraction.

2.3 Extract Preparation

Coffee cherry was fractionally macerated using hexane for 48 h, in 3 cycles. The residue was then dried in the hot air oven at 50 °C for 24 h and extracted with ethyl acetate using the similar method as hexane extraction. After ethyl acetate extraction, the residue was dried in the hot air oven and extracted again with 95 (% v/v) ethanol. Each sample solution was filtrated through Whatman® filter paper No.1 and evaporated using a rotary evaporator. All coffee cherry extracts were stored in well tight amber container at 4 °C until usage. The obtained extracts from coffee cherry were named as CH (hexane extract), CEA (ethyl acetate extract) and CE (ethanol extract), respectively.

2.4 *In Vitro* Antioxidant Activity of Coffee Cherry Extracts

2.4.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The scavenging activity of each extract on DPPH radical was adapted from Thaipong et al. (2006) [8]. Each extract (5 mg/mL) was dissolved in ethanol and serially diluted to give concentrations of 2500, 1250, 625 and 312.5 μg/mL. Each 20 mL of sample solution was mixed with 180 mL of DPPH solution and incubated for 30 min in the dark at room temperature. An absorbance of sample was measured at 520 nm using microplate reader (Backman coulter, DTX 880 multimode detector, Austria). Chlorogenic acid and caffeine were used as standards. Percentage of inhibition (%) was calculated using the following equation:

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

Where Ac is an absorbance of control and As is an absorbance of sample. The graph of each extract plotted between % inhibition (Y) and concentration (X) was utilized for calculating 50% inhibitory concentration (IC_{50}) value.

2.4.2 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was adapted from Benzie and Strain (1996) [9]. FRAP reagent consisted of 0.3 M acetate buffer (pH 3), 10 Mm TPTZ dissolved in 40 mM of 37% HCl (v/v) in deionized water, and 20 mM ferric chloride solution with a ratio of 10:1:1. The concentration of each extract was 1 mg/mL dissolving in 95% (v/v) ethanol and 20 μL of each sample was added in 96-well plate and reacted with 180 μL of FRAP reagent. The mixture was incubated at room temperature for 5 min. The dark blue color solution was measured at 595 nm by multimode detector. Ferrous sulfate was used as standard with equation:

$$y = 0.1405x + 0.0287$$

Y is an absorbance at 595 nm and X is concentration of $FeSO_4$ ($r^2 = 0.9926$). The results of ferric reducing antioxidant power were reported as FRAP value by the following equation:

FRAP value (mM $FeSO_4$ /g)
= [(A-B) – 0.0287] / 0.1405

A is an absorbance of control of sample and B is an absorbance of blank of sample.

2.4.3 Lipid peroxidation inhibition assay

The lipid peroxidation process based on the ferric-thiocyanate method was detected by following the method of Osawa and Namiki (1981) [10]. Extracts and standards with concentration 20 mg/mL were dissolved in 95% (v/v) ethanol. Amount of 50 μL of each extract was mixed with 50 μL of linoleic acid in 50% DMSO (v/v), 50 μL of 5 mM ammonium thiocyanate (NH_4SCN), and 50 μL of 2 mM ferrous chloride ($FeCl_2$). The mixture was placed in a hot air oven at 37 ± 0.5 ºC for 1 h and then the reaction was investigated. The control was prepared as same as the sample without linoleic acid. Chlorogenic acid and caffeine were used as standards. The absorbance of each extract was detected at 490 nm using multimode detector. The percentage of inhibition was calculated by the following equation:

% inhibition = [(Ac – As) /Ac] x 100

Ac is an absorbance of control and As is an absorbance of the sample.

2.5 *In Vitro* Anti-Tyrosinase Activity of Coffee Cherry Extracts

The anti-tyrosinase activity of each extract was determined using the modified dopachrome method with L-tyrosine and L-dopa as substrates that modified from Khunkitti et al. (2012) [11]. The experiment was conducted in a 96-well plate. Each extract was dissolved in ethanol with different concentrations (5, 2.5, 1.25, 0.625, 0.312, 0.156 mg/mL). Briefly, each 70 μL of sample was mixed with 70 μL of phosphate buffer solution and 70 μL of mushroom tyrosinase enzyme (50 units/mL in phosphate buffer pH 6.5) in a 96-well plate. Then, the mixture was incubated at 25 °C for 10 min before adding 70 μL of 2.5 mM L-tyrosine or 2.5 mM L-dopa in phosphate buffer. Finally, the generated dopachrome was determined after 20 min of incubation at 25 °C by the absorbance measurement at 450 nm using multimode detector. The results were compared to the positive controls including kojic acid and vitamin C in the concentration range of 0.031-1 mg/mL. The anti-tyrosinase activity of each sample expressed as percentage tyrosinase inhibition was calculated using the following equation.

% inhibition = [(Aa-Ab)/Aa] × 100

Aa is an absorbance at 450 nm without test sample, Ab is an absorbance at 450 nm with test sample.

2.6 Determination of Total Phenolic Content

Coffee cherry extracts were determined total phenolic content by Folin-Ciocalteu assay [12]. Each sample was dissolved in ethanol (1 mg/mL) and then the 500 μL of sample was transferred into a test tube, mixed with Folin-Ciocalteu reagent and Na_2CO_3 7.5% w/v in distilled water. The mixture was incubated for 30 min in the dark condition. The absorbance was measured at 765 nm using a spectrophotometer (Shimadzu UV-Vis 2450, Japan). The concentration of total phenolic content in each extract was calculated as gallic acid equivalent (GAE), in milligram gallic acid/ gram of sample.

2.7 Matrix Metalloproteinase Activity Determination by Gel Electrophoresis

The effect of each coffee cherry extract on matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) was evaluated by gel electrophoresis.

The NIH-3T3 cells were treated with each coffee cherry extract (1 mg/mL) in DMSO at 37 °C and 5% CO_2 for 48 h. The expression of MMP-2 and MMP-9 was carried out by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, each extract solution (extract dissolved in DMSO) was mixed with 0.025% (w/v) bromophenol blue, 4% (v/v) SDS, 25% (v/v) of 0.5 M Tris-HCl (pH 6.8) and 20% (v/v) glycerol in distilled water. The sample mixture was then loaded into each well of sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) containing 0.1% (w/v) gelatin. After that voltage was applied directly to the Tris/glycine/SDS running buffer of SDS-PAGE. After separation process, SDS-PAG was removed and soaked in reaction buffer, composed of 0.1 M Tris-HCl (pH 8.0), 5% (v/v) Triton X-100, and 200 mM NaCl, at 37 °C for 1 h. One milliliter of 1.0 M $CaCl_2$ was then added to the reaction buffer and incubated again at 37 °C for 23 h. After incubation, the gel was washed with DI water and incubated in fixing buffer, composed of 50% (v/v) methanol and 12% (v/v) acetic acid in DI water, at 37 °C for 30 min. The gel was then washed with DI water and stained by staining buffer, composed of 0.025 g of Coomassie blue, 40% (v/v) methanol, and 7% (v/v) acetic acid in DI water, for 1 h. After staining process, the gel was then destained by destaining buffer, composing of 40% (v/v) methanol and 7% (v/-v) acetic acid in in distilled water. In final process, the expression of MMP-2 and MMP-9 was then calculated by ImageJ 1.51J8 program (Wayne rasband, NIH, USA).

2.8 Hyaluronidase Activity Determination by Gel Electrophoresis

The expression of hyaluronidase was also carried out using SDS-PAGE. Each coffee cherry extract (1 mg/mL) was incubated with 0.1 g of hyaluronidase from bovine testis dissolved in 20% (v/v) of 0.15 M NaCl at 37 °C for 48 h. After incubation, each sample (5 µL) was mixed with 100 µL of dye solution that consists of 0.025% w/v bromophenol blue, 4% (v/v) SDS, 25% (v/v) of 0.5 M Tris-HCl, pH 6.8, and 20% (v/v) glycerol in distilled water and the sample mixture (20 µL) was loaded into each well of SDS-PAG containing 0.17% (w/v) hyaluronic acid. After that voltage was applied directly to the Tris/glycine/SDS running buffer of SDS-PAGE. After the separation, SDS-PAG was removed and soaked with the washing buffer, composing of 50 mM Tris-HCl (pH 8.0), 2.5% (v/v) Triton X-100, and 100 mM NaCl in DI water, at 37 °C for 1 h. The gel was then incubated in reaction buffer, composing of 10% (v/v) of 0.2 M acetate buffer (pH 5.0) and 90% (v/v) of 0.15 M NaCl, with continuously shaking at 60 rpm at 37 °C for 16 h. After incubation, the gel was washed with DI water and stained by staining buffer, composed of 0.5% (w/v) alcian blue 8GX and 3% (v/v) acetic acid in DI water, for 1 h. The gel was then destained by destaining buffer, composed of 50% (v/v) methanol and 1.0% (v/v) acetic acid in DI water and the expression of hyaluronidase was calculated by using ImageJ 1.51J8 program (Wayne rasband, NIH, USA).

2.9 Effects of Coffee Cherry Extracts on Cell Viability of The Mouse Fibroblast Cell Line, NIH-3T3, Measured by the MTT Assay

Cell viability of treated mouse fibroblast cell line, NIH-3T3, was evaluated by MTT assay. MTT assay is a colorimetric assay based on the ability of mitochondrial succinate dehydrogenase activity in viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; MTT] to a blue formazan crystal. Briefly, the NIH-3T3 cells (1×10^4 cells) were seeded into 96-well plate and cultured at 37 °C 5% CO_2 for 24 h, followed by treatment with different concentrations (0.8-800 µg/mL) of

the cherry coffee extracts (CE and CEA) for 48 h. Fifteen microliters of 5 mg/mL MTT was added into each well and incubated at 37 °C, 5% CO_2 for 4 h. After incubation, the supernatant was removed and 200 μL DMSO was added to resolve the formazan crystal. The absorbance was measured at 578 nm using an AccuReaderTM microplate reader (Metertech Inc., Taipei, Taiwan) and the absorbance at 630 nm was used as reference. All experiments were performed in triplicate. The absorbance of vehicle control was considered as 100% cell viability. The percentage of cell viability in each treatment was calculated as followed:

% cell viability
= (absorbance of treatment x 100)
/absorbance of vehicle control

2.10 Effects of Coffee Cherry Extracts on Cell Viability of H_2O_2-Treated NIH-3T3 Cells Measured by the MTT Assay

In order to investigate the inhibition activity of coffee cherry extracts (CE and CEA) on H_2O_2-treated NIH-3T3, NIH-3T3 cells were co-culture with 0.002% H_2O_2 and 8 μg/mL coffee cherry extracts and cell viability was evaluated by MTT assay. Briefly, the NIH-3T3 cells (1 x 10^4 cells) were seeded into 96-well plate and cultured at 37 °C, 5% CO_2 for 24 h, followed by treatment with 0.002% H_2O_2 in condition with or without 8 μg/mL cherry coffee extracts for 48 h. Fifteen microliters of 5 mg/mL MTT was added into each well and incubated at 37 °C, 5% CO_2 for 4 h. After incubation, the supernatant was removed and 200 μL DMSO was added to resolve the formazan crystal. The absorbance was measured at 578 nm using an AccuReader™ microplate reader and the absorbance at 630 nm was used as reference. All experiments were performed in triplicate. The absorbance of vehicle control (free H_2O_2) was considered as 100% cell viability and the H_2O_2-treated NIH-3T3 cells was used as positive control. The percentage of cell viability in each treatment was calculated as described previously.

2.11 Irritation Test by Hen's Egg Test on The Chorioallantoic Membrane (HET-CAM)

Irritation score was evaluated by using the hen's egg test chorioallantoic membrane (HET-CAM) following the method used by Chaiyana et al. (2017) [13]. Hen eggs were obtained from the Faculty of Agriculture, Chiang Mai University, Thailand. All eggs were incubated for 7 days in the hatching chamber with 37.5 ± 0.5 °C, humidity 55 ± 7% before start experiment. The eggshell was opened and the white egg membrane was removed. The thirty microliters of CE or CEA solution in DI water (10 mg/mL) was dropped to the CAM. Changing of the membrane and its blood vessel was observed including hemorrhage, lysis, and coagulation after adding the sample for 5 min. 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 first vascular hemorrhage was detected, t(l) is the time (s) when first vascular lysis was detected, and t(c) is the time (s) when the first 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.

2.12 Statistical Analysis

All data were presented as a mean ± standard deviation (SD). Statistical significance was assessed by the one-way analysis of variance (ANOVA) followed by post-hoc tests using the SPSS statistic version 17.0 program. P value less than 0.05 ($p < 0.05$)

was considered statistically significant.

3. RESULTS AND DISCUSSION

All of the coffee cherry extracts were dark brown and semisolid. Among the three solvents, it was found that the yield of the ethanolic extract (CE) (1.99%) was higher than that of the ethyl acetate extract (CEA) (1.78%) and the hexane extract (CH) (0.74%).

3.1 *In vitro* Antioxidant Activity and Total Phenolic Content of Coffee Cherry Extracts

Antioxidant activities of each extract assessed by the DPPH, FRAP and lipid peroxidation inhibitory assays are shown in Table 1. There are many assays for the assessment of antioxidant activity that protects against free radicals. For the DPPH assay, the CEA exhibited strong DPPH radical scavenging capacity with an IC_{50} value of 35.65 ± 1.35 μg/mL, similar to chlorogenic acid and followed by CH and CE, respectively. Chlorogenic acid exhibited stronger free radical scavenging activity than the extracts presumably due to the hydroxyl group in its structure. Chlorogenic acid is the major polyphenol compound in coffee, isolated from the leaves, beans and fruits of coffee and other plants [14]. Therefore, the coffee cherry extracts showed scavenging capacity presumably due to the potential of chlorogenic acid in the extracts. For the FRAP assay, the FRAP value of CE was 141.84 ± 0.71 mM $FeSO_4$/g, followed by CEA and CH, respectively. Coffee cherry extracts exerted antioxidant ability by reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) through electron donation [15]. In the results of the DPPH and FRAP assays, the ethanolic and ethyl acetate extracts presented better antioxidant property than the hexane extract. This may be due to the phenolic compounds in the extracts, which were extracted by polar or semi-polar solvents. For the lipid peroxidation inhibitory assay, lipid peroxidation occurs in cell membranes when a hydroxyl radical abstracts an electron from an unsaturated fatty acid [16,17]. In this study, the percentage inhibition of extracts and caffeine on lipid peroxidation inhibition were not detected, except in chlorogenic acid, which showed the percentage inhibition as 34.12 ± 1.87. It can thus be assumed that chlorogenic acid can inhibit the oxidation reaction of unsaturated fatty acids by donating hydrogen atoms to stop the chain reaction.

Table 1. Antioxidant activities determined by DPPH, FRAP and lipid peroxidation inhibitory assays and the total phenolic content of coffee cherry extracts.

Samples	DPPH assay	FRAP assay	Lipid peroxidation inhibitory assay	Total phenolic content (mg GAE/g extract)
	IC_{50} (μg/mL)	FRAP value (mM $FeSO_4$/g)	% inhibition	
CH	112.10 ± 0.57	11.77 ± 0.04	ND	ND
CEA	35.65 ± 1.35	100.52 ± 0.08	ND	301.96 ± 4.05
CE	182.98 ± 1.42	141.84 ± 0.71	ND	222.88 ± 5.11
Caffeine	ND	37.00 ± 0.2	ND	-
Chlorogenic acid	10.51 ± 0.5	234.50 ± 0.5	34.12 ± 1.87	-

ND = No detectable

The total phenolic content of cherry coffee extracts was determined by the Folin-Ciocalteu colorimetric method compared with gallic acid. Phenolic compounds naturally found in coffee are a group of phenolic acids, such as chlorogenic acid (5-caffeoylquinic acid), epicatechin, catechin, rutin, protocatechuic acid and ferulic acid [4]. The total phenolic content of the coffee cherry extracts is shown in Table 1. The ethyl acetate extract showed the highest total phenolic content, followed by the ethanolic and hexane extracts, respectively. The total phenolic content of CEA and CE was 301.96 ± 4.05 and 222.88 ± 5.11 mg GAE/g extract, respectively. These results correspond well to antioxidant activity since the ethanolic and ethyl acetate extracts that contained the highest total phenolic content also showed good antioxidant activity.

3.2 *In Vitro* Anti-Tyrosinase Activity of Coffee Cherry Extracts

The results from the study of the anti-tyrosinase activity of coffee cherry extracts are shown in Table 2. The CEA presented the highest anti-tyrosinase activity with the half maximal inhibitory concentration (IC_{50}) value at 0.23 ± 0.01 mg/mL when using L-tyrosine as a substrate and also presented the IC_{50} value at 0.98 ± 0.02 mg/mL when using L-dopa as a substrate. Furthermore, the CE also inhibited tyrosinase activity when using L-tyrosine and L-dopa as substrates. The extract exhibited the IC_{50} value at 1.77 ± 0.22 mg/mL and 4.59 ± 0.15 mg/mL, respectively. Although, kojic acid and vitamin C showed better anti-tyrosinase activity than the coffee cherry extracts. The results demonstrated that the coffee cherry extracts have the potential to inhibit tyrosinase, which could prevent hyperpigmentation such as lentigo, freckles and dark spots. Therefore, they can be applied in natural products as a brightening agent.

3.3 Matrix Metalloproteinase and Hyaluronidase Activities Determination by Gel Electrophoresis

The effects of coffee cherry extracts on matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) on NIH-3T3 cells were evaluated by gel electrophoresis. The inhibitory activities of coffee cherry extracts against MMP-2 are shown in Figure 1. The ethyl acetate extract possessed the highest inhibitory activity against MMP-2, followed by the ethanolic and hexane extracts, respectively. The standard compounds, chlorogenic acid, caffeic acid and caffeine, also inhibited the activity of MMP-2. MMPs are zinc-containing endopeptidases that are related to the degradation

Table 2. Half maximal inhibitory concentration (IC_{50}) value of standards and extracts.

Samples	IC_{50} (mg/mL)	
	L-tyrosine	L-dopa
Kojic acid	0.003 ± 0.03	0.024 ± 0.03
Vitamin C	0.048 ± 0.04	0.56 ± 0.02
CH	ND	ND
CEA	0.23 ± 0.01	0.98 ± 0.02
CE	1.77 ± 0.22	4.59 ± 0.15

ND = No detectable.

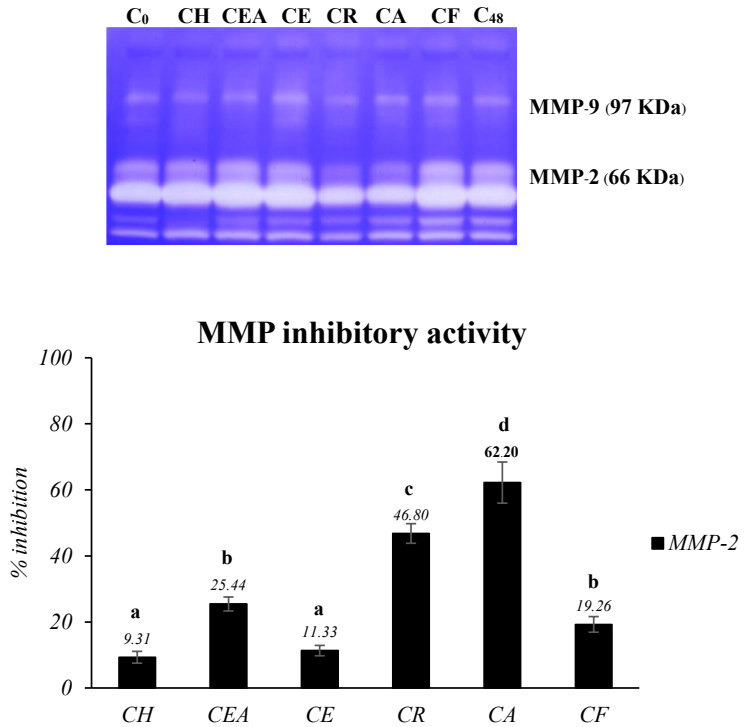

Figure 1. Effects of hexane extract (CH), ethyl acetate extract (CEA), ethanolic extract (CE), chlorogenic acid (CR), caffeic acid (CA) and caffeine (CF) on MMP-2 inhibitory activity in NTH-3T3 cells. The data present the mean ± SD of the three independent experiments. Different letters indicate significant difference ($p < 0.05$).

of the different components of the extracellular matrix proteins such as collagen, fibronectin, elastin, and proteoglycans [18]. MMPs can be categorized into five main subgroups based on their substrate specificity and structural organization. However, MMP-1 (collagenase), MMP-2 (gelatinase A), MMP-3 (Stromelysin-1), and MMP-9 (gelatinase B) are related to the degradation of collagen and elastin [19]. The increasing expression of MMPs leads to an increase of collagen breakdown and generates wrinkle formation and repeated skin damage [20]. However, the results showed that this elevated MMP-2 expression was suppressed by coffee cherry extracts. In addition, chlorogenic acid and caffeic acid are well-known plant polyphenols, and they are found in coffee extracts. The current results suggest that chlorogenic acid could reduce the activity of MMP-2 and are in accordance with the previous reports that it could inhibit MMP inhibitors in clinical studies [21]. Therefore, coffee cherry extracts have the potential to be used as a new natural resource in cosmeceutical applications that can prevent oxidative stress-induced premature skin aging. The aging of skin is also associated with the loss of skin moisture. Hyaluronan or hyaluronic acid is a linear glycosaminoglycan disaccharide that is involved in skin moisture. It can bind and retain water molecules within the skin [22]. It is found in both the dermis and epidermis. Hyaluronidases is a key enzyme that catalyzes the degradation of hyaluronic acid into smaller hyaluronic acid fragments [23]. The reduction

of hyaluronic acid in the skin leads to changes of the skin structure and it can generate aging skin. The inhibitory activities of coffee cherry extracts against hyaluronidase investigated by gel electrophoresis are shown in Figure 2. Hexane and ethyl acetate extracts could inhibit hyaluronidase with the percentage inhibition of more than 90%, whereas ethanolic extract could exhibit hyaluronidase approximately 50%. The hyaluronidase inhibitory activities of coffee cherry extracts were significantly higher than chlorogenic acid, caffeic acid and caffeine. Therefore, the coffee cherry extracts that could inhibit hyaluronidase might be useful for anti-aging agent for skin care product. The previous reports presented that hyaluronidase inhibitory activity is characteristic of quercetin derivatives (rutin and quercetin 3-*O*-β-glucuronide) and the phenolic acids (chlorogenic and protocatechuic) [24,25,26]. The hyaluronidase inhibitory effects of coffee cherry extracts may be derived from these compounds.

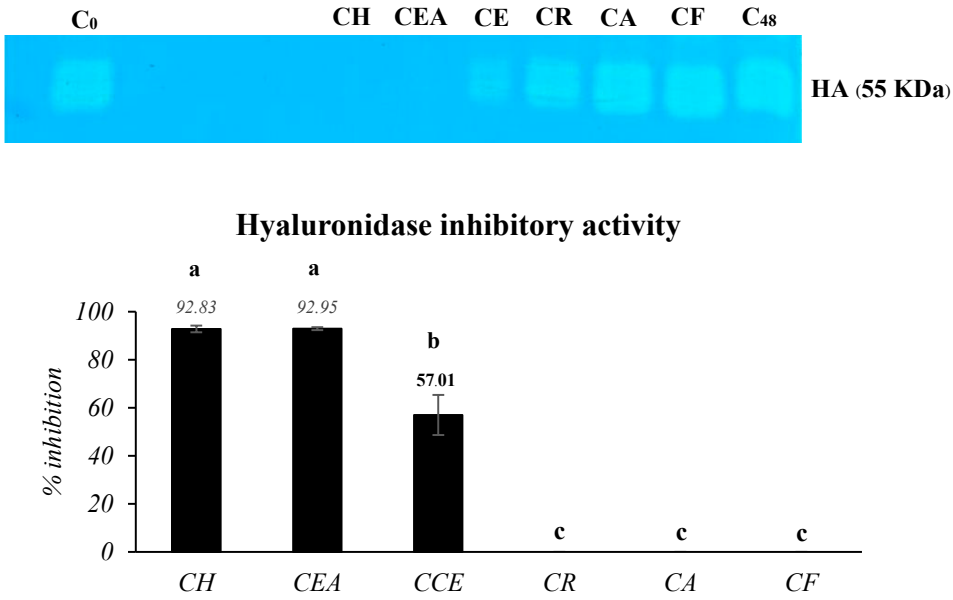

Figure 2. Effects of hexane extract (CH), ethyl acetate extract (CEA), ethanolic extract (CE), chlorogenic acid (CR), caffeic acid (CA) and caffeine (CF) on the hyaluronidase inhibitory activity in NIH-3T3 cells. The data presentthe mean ± SD of three independent experiments. Different letters indicate significant difference ($p < 0.05$).

3.4 Effects of Coffee Cherry Extracts on Cell Viability of The Mouse Fibroblast Cell Line, NIH-3T3, Measured by The MTT Assay

The CE and CEA were selected for further examination because they showed good activities based on the antioxidant, anti-tyrosinase and anti-aging assays. To investigate the effects of the coffee cherry extracts on cell viability of the mouse fibroblast cell line, NIH-3T3, the cells were treated with different concentrations of coffee cherry extracts (CE and CEA) and cell viability was measured using the MTT assay. The results are shown in Figure 3. The CE

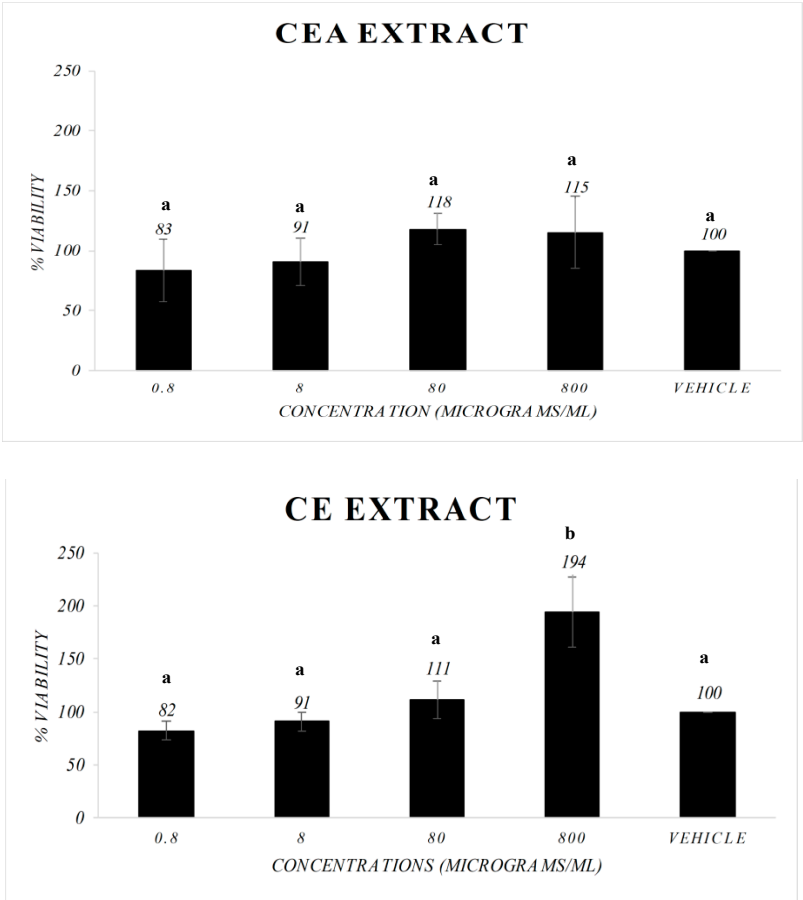

Figure 3. Effects of CEA and CE extracts on cell viability of the mouse fibroblast cell line, NIH-3T3, measured by the MTT assay. Different letters indicate significant difference ($p < 0.05$).

and CEA did not have any cytotoxic effects on the proliferation of NIH-3T3 due to the high percentage of cell viability (80-100%). Only the 800 μg/mL concentration of the CE exhibited the growth stimulatory effect on NIH-3T3 cells that it was significantly different ($p < 0.05$) from the other concentrations. It was presumably due to amount of chlorogenic acid in the extract. The results are related with the study of Tsuruya et al. (2014) [27], in which chlorogenic acid could stimulate the proliferation of mouse fibroblasts [27].

3.5 Effects of Coffee Cherry Extracts on Cell Viability of H_2O_2-Treated NIH-3T3 Cells Measured by The MTT Assay

Oxidative stimulation was induced by 0.002% hydrogen peroxide (H_2O_2) on NIH-3T3 cells. It is well-known that oxidative stress plays an important role in the skin aging process. The mechanism of oxidative stress by H_2O_2 in skin cells involves reactive oxygen species generation [28]. Therefore, hydrogen peroxide results in the aging of skin and cell damage. In order to investigate the percentages of cell viability of these coffee cherry extracts

(CE and CEA), they were measured in the H_2O_2-induced oxidative stress in NIH-3T3 cells using the MTT assay. After incubation of cells with 8 μg/mL of the CE and CEA, protection against H_2O_2-induced cytotoxicity in NIH-3T3 cells, vehicle as well as the H_2O_2 free vehicle was shown in Figure 4. When cells were treated with the CE, the result showed 24% in cell viability. The CE also exerted a better protective effect than H_2O_2-treated cells (positive control) and the CEA with the same concentration of 1 mg/mL with significant difference ($p < 0.05$). Therefore, these results suggest that coffee cherry extracts might tend to protect cells from oxidative stress when increase the concentration of the extracts. In addition, phenolic compounds in coffee cherry extracts and chlorogenic acid presumably act as free radicals scavenging agents that prevent cell damage from H_2O_2 [29].

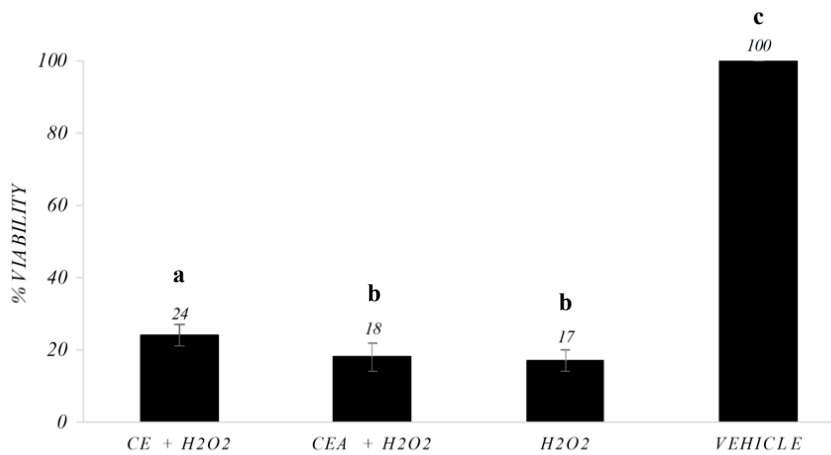

Figure 4. Effects of coffee cherry extracts (8 μg/mL) on cell viability of H_2O_2-treated NIH-3T3 cells (H_2O_2 0.002%) measured by the MTT assay. Different letters indicate significant difference ($p < 0.05$).

3.6 Irritation test by Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM)

The results of the irritation test using the HET-CAM assay are shown in Table 3. The positive control was 1% w/v SLS in deionized water that could induce an irritation effect on chorioallantoic membrane (CAM) as shown in Figure 5. It indicated severe irritation with the irritation index (IS) score of 11.38 ± 0.03. In contrast, the CE and CEA (10 mg/mL) exhibited non-irritation similar to the negative control (0.9% w/v NaCl) as seen in Figure 6. The appearance of blood vessels before and after treatment with the CE and CEA are presented in Figure 7, which shows that they did not harm blood vessels and capillaries on the CAM surface. Therefore, the CE and CEA can be considered as safe for further studies in animal model and volunteers. The HET-CAM model is used for predicting the irritation effect on the conjunctiva, and it can confirm the safety of the tested substances. It can also evaluate the irritation of many types of cosmetics formulations [30].

Table 3. Irritation scores and irritation assessment from the HET-CAM assay.

Sample	Irritation index (IS) score	Irritation level
CE	0.0	Non-irritation
CEA	0.0	Non-irritation
Negative control (0.9% w/v NaCl)	0.0	Non-irritation
Positive control (1% w/v SLS)	11.38 ± 0.03	Severe irritation

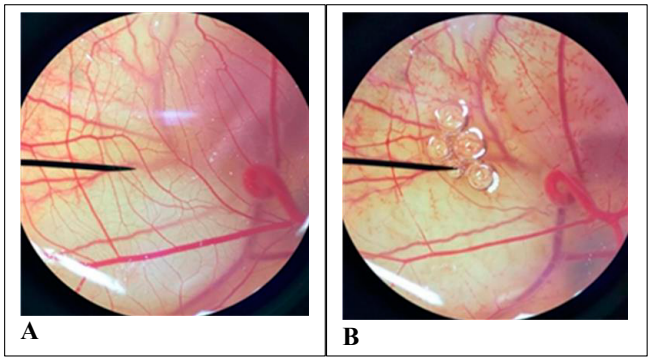

Figure 5. Appearance of blood vessels before (A) and after treatment with 1% w/v sodium lauryl sulfate for 5 min (B).

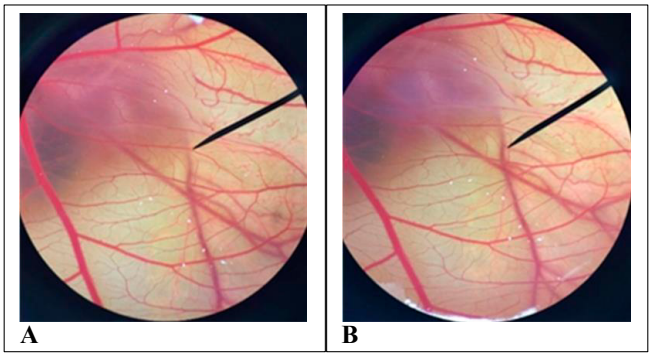

Figure 6. Appearance of blood vessels before (A) and after treatment with 0.9% w/v sodium chloride for 5 min (B).

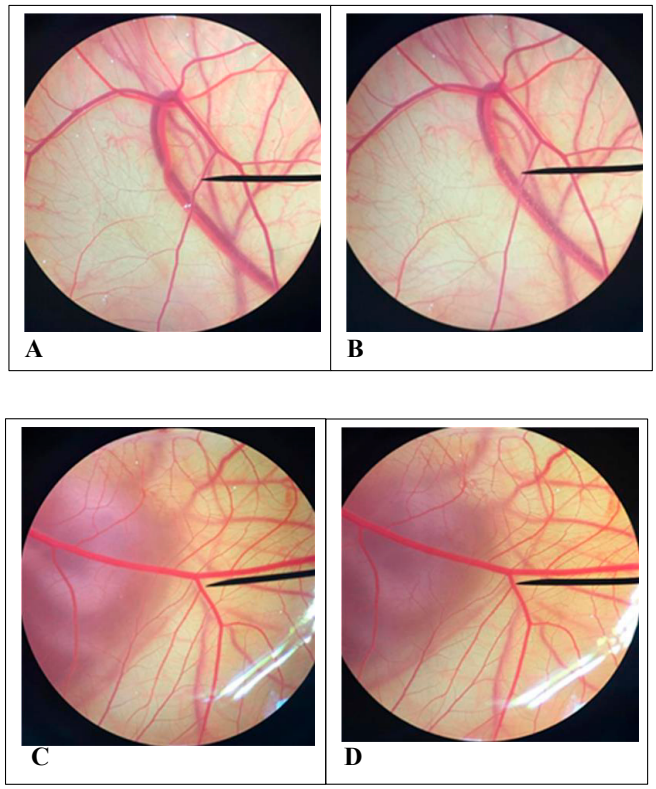

Figure 7. Appearance of blood vessels before (A and C) and after treatment with ethyl acetate coffee cherry extract for 5 min (B) and after treatment with ethanolic coffee cherry extract for 5 min (D)

4. Conclusion

This study focused on investigating the biological activities and safety of coffee cherry extracts. Among the various extracts, the ethyl acetate extract possessed the most potent antioxidant, anti-tyrosinase and anti-aging activities. In addition, it might tend to protect H_2O_2 induced NIH-3T3 cell damage and also presented non-toxicity toward cells. Furthermore, it did not cause irritation in the HET-CAM, which confirmed its safety for further study in vivo. It showed high phenolic content that was responsible for those biological activities that were related to antioxidant and anti-tyrosinase properties, including having only mild inhibition of MMPs and potential for prevention of the degradation of hyaluronic acid. Therefore, this coffee cherry extract could be an attractive natural extract for further development of whitening or anti-aging products. Moreover, this can be a way to develop the value of arabica coffee waste into a value product that can be applied in the cosmetics industry.

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