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EVALUATION OF ANTIOXIDANT AND ANTI-AGING EFFICACIES OF *COFFEA ROBUSTA* EXTRACT ON HUMAN FIBROBLAST

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ABSTRACT

The extract of *Coffea robusta* was evaluated via the antioxidant and anti-aging efficacies on human dermal fibroblast. *Coffea robusta* green beans harvested in Buon Ma Thuot - Vietnam were roasted and extracted by hot water. The anti-oxidant activity of the extract was measured by the DHHP assay. Additionally, the extract was dosed to fibroblast and proteome analysis using 2-D was conducted to observe the changes of proteins involving in aging process. The results show that the extract is a strong anti-oxidant factor with low IC_{50} value for DPPH scavenging (33.2 mg/L), and significant up-regulation of 5 natural antioxidant enzymes (GPX2, GCLM, PRDX4, GSTA1 and NQO1), as well as down-regulation of 2 supporting-free-radicals proteins (NF-kB1 and TNF-alpha) of human fibroblast. Furthermore, the extract is a potential anti-aging factor with the down-regulation of 4 proteins related to the degradation of elastin and dermal extracellular matrix (CELA2B, CELA3A, CELA3B and MMP3).

Keywords: anti-aging, anti-oxidant, *Coffea robusta*, human fibroblast.

1. INTRODUCTION

Coffee has been suggested that being the beverage with high antioxidant capacity [1], which is greater than most other beverages [2]. There are many assays for measuring antioxidant capacity of coffee which have been reported [1-6], however most of them are based on different chemical reactions and less of them are *in vitro* and *in vivo* tests. Vicente SJ. et al investigated the effect of coffee brew on the activity of antioxidant enzymes in rats and found that coffee could be considered an important alternative against oxidative stress [6]. Svilaas A. et al. determined the contribution of various food groups to total antioxidant intake, and to assess the correlations of the total antioxidant intake from various food groups with plasma antioxidants in human and they found that the single greatest contributor to the total antioxidant intake was coffee [5]. Nevertheless, there has not been yet any study reporting about coffee antioxidant capacity on human fibroblast of which results are very useful for cosmeceutical science and application.

2. MATERIALS AND METHODS

2.1. Materials

Normal human fibroblast cells (NF) were maintained in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotic (AA). Experimental cells were prepared at passage 7^{th} and at the density of 3×10^4 cells/ml. All cells were incubated at 37 ^oC in 5% CO_2.

Coffea robusta Extract: *Coffea robusta* green beans harvested in Buon Ma Thuot – Viet Nam were roasted at 230 ^oC in 15 minutes. The roasted coffee powder was then extracted by hot distilled water (DW) at the ratio 1:10 in about 3 h. The clear liquid was collected for further experiments.

2.2. The DPPH radical scavenging assay

DPPH (2,2-Diphenyl-1-picryl-hydrazyl) radical scavenging assay was used to determine the antioxidative activity of coffee extract. 100 µl of the extracts of *Coffea robusta* at concentrations of 1, 10, 50, 100, 500 and 1000 mg/L was placed into each well of 96-well plate. 100 µl of 0.15 mM methanolic solution of DPPH were added into every well. The plate was shaken vigorously and allowed at room temperature in 30 minutes. The control was prepared as above without any extract and DW was used for the baseline correction. Optical density (OD) of the samples was measured at 490 nm using iMark™ microplate absorbance reader (Bio-Rad). Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\%\ \text{radical scavenging activity} = \left(1 - \frac{OD_{Sample} - OD_{Blank}}{OD_{Control}}\right) * 100\%$$

2.3. MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is used to determine cytotoxicity of coffee extract for the viability and growth of human fibroblast cells. 100µl of experimental cell solution was plated into each well of 96-well culture plate and incubated at 37 ^oC for 24 h in 5% CO_2 incubator. After treatment of cells for 48 h by coffee extract at concentrations of 1, 5, 10, 50 and 100mg/L in DMEM containing 1% AA, experimental media are removed and the cells are incubated with 50µl basal medium containing 2.0 mg/ml MTT in CO_2 incubator at 37 ^oC for 3 h. The medium is aspirated, and the formazan product is solubilized with 200 µl Dimethyl Sulfoxide (DMSO) every well. Absorbance at 595 nm was measured for each well using iMark™ microplate absorbance reader (Bio-Rad).

2.4. Protein extraction

Experimental NF cells were plated into 100 mm-petri dish. After 24 h being incubated at 37 ^oC and 5% CO_2, the cells were treated by *Coffea robusta* extract at the non-toxic concentration and incubated in 48 h more. Cells were collected by Trypsin EDTA solution and washed by 10-time diluted PBS. Proteins were extracted from the cell pellet by adding 100 µl Lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 1% DTT, 2% Carrier ampholyte, 4% PIC, 0.002% Bromo Phenol Blue), sonicating in 1 min and incubating at 30 ^oC in 5 h, centrifuging at 13400 rpm in 1 min and finally collecting the clear solution. The amount of total protein extracted was measured via the modified Bradford method.

2.5. Two-dimensional electrophoresis

Isoelectric focusing (IEF): The extracted proteins were loaded into 24-cm-long linear pH 3-10 immobilized pH gradient strips which were rehydrated in about 12 h before by 500 µl Rehydration solution (7 M Urea, 2 M Thiourea, 4% CHAPS, 1% DTT, 2% Carrier ampholyte, 10% glycerol, 0.002% Bromo Phenol Blue) for every strip. Proteins were isoelectrically focused using an Ettan IPGphor 3 (GE Healthcare, USA) system at 500 V for 24 min, then ramped to 10000 V for 1 h and then kept at 10000 V for 8 h.

Prior to the second dimension separation, disulfide bonds were reduced by incubating the immobilized pH gradient strips for 15min with 1% DTT in equilibration buffer (7 M Urea, 2 M Thiourea, 2% SDS, 50 mM Tris-HCl, 30% glycerol, 0.002% Bromo Phenol Blue) and free SH-groups were alkylated by treated with 2.5% Iodoacetamide in equilibration buffer. Second-dimension (2-D): Following equilibration, the strips were transferred to 12.5% acrylamide homogenous gels (25 × 20 cm) and run at 40 mV/gel in about 5 h. Gels were fixed and stained by nitrate silver staining method.

Bio-Rad 2-D SDS-PAGE Standards No.16100320, a mixture of seven proteins including myoglobin, trypsin inhibitor, CA, GAPDH, actin, BSA, and conalbumin (17500–76000 molecular weight range, isoelectric point 4.5–8.5), was utilized as the internal molecular weight (MW) and isoelectric point (pI) markers on both control NF gel and treated NF gel.

2.6. Spot detection and quantification of Protein Expression

The stained gels were scanned, then detected and quantified expressed protein spots using ImageMaster™ 2D platinum software. Firstly, the seven internal standard proteins were marked and matched manually between control NF gel and treated NF gel. After that, the molecular weight and isoelectric point of all spots on both gels were automatically identified based on the internal standards using ImageMaster™ 2D platinum software. The relative intensities of the spots were used for a comparison between the control NF gel and treated NF gel. All spots considered by the software as significantly regulated were visually checked, selected and a pick list was created. The commonly differentially expressed spots (2-fold increase or decrease) were further identified by MALDI-TOF/TOF MS.

The area and pixel intensity of each spot of interest were measured, enabling calculation of individual spot volumes, which were expressed as percentage of the integrated spot volume for the entire gel.

2.7. Statistical analysis

The statistical comparisons between control and treated NF protein spots were assessed by Student's *t*-test (ImageMaster™ 2D platinum software, with $p < 0.05$ considered to be significant).

3. RESULTS AND DISCUSSION

3.1. DPPH scavenging activity

Roasted *Coffea robusta* extracted by hot water was tested for DPPH Scavenging activity and compared to the green *Coffea robusta* extract. The results were shown in Table 1. The

calculated IC50 of *Coffea robusta* extracts were 194.37 mg/L for green extract and 33.20 mg/L for roasted extract. This result showed that the anti-oxidant activity of coffee extract is in concentration-dependent manner. Moreover, *Coffea robusta* extract has stronger anti-oxidant efficacy after roasting, similar with previous study results [3, 4, 7].

Table 1. DPPH scavenging activity of *Coffea robusta* extracts at different concentrations.

Conc. (mg/L)	**0.5**	**5**	**25**	**50**	**250**
Green bean	7.20 ± 1.54	9.40 ± 2.73	14.50 ± 1.91	39.10 ± 2.15	54.20 ± 1.84
Roasted bean	10.46 ± 1.98	20.92 ± 1.27	36.60 ± 2.88	77.45 ± 2.03	90.17 ± 1.31

3.2. Toxicity on human fibroblast

The result of MTT assay displayed in Fig. 1 showed that toxicity of roasted *Coffea robusta* extract on NF depended on treating extract concentration. At treating extract concentrations from 0 to 10 mg/L, the cells were still alive. At 50 and 100 mg/L of the treating extract, about 20% and 35% of cells were killed respectively. Therefore, 10 mg/L of roasted *Coffea robusta* extract was chosen to treat NF in 2-D experiments because of its no toxicity.

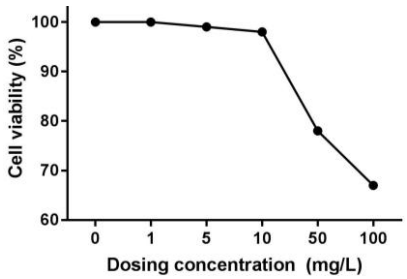

Figure 1. Cell viability (%) of human fibroblast treated by *Coffea robusta* extract.

3.3. Two-dimension proteome analysis

Two-dimension proteome analysis was conducted on control NF and treated NF which was dosed by 10 mg/L of roasted *Coffea robusta* extract. 2D-PAGE images of proteins extracted from control NF and treated NF were displayed in Fig. 2. The changes of antioxidant and anti-aging related proteins were observed by comparing the control and treated 2-D gels. There were 717 and 612 detected spots on control gel and treated gel, respectively. Among them, 447 spots were matched in which 160 spots were up-regulated and 287 spots were down-regulated.

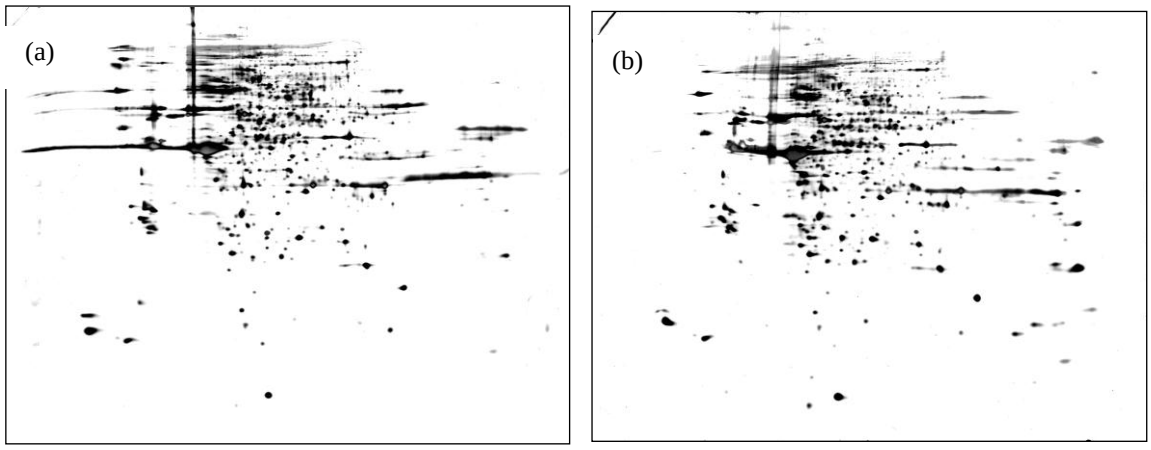

Figure 2. 2D-PAGE images of proteins extracted from human fibroblast: (a) Control NF, (b) Treated NF.

Based on isoelectric point and molecular weight, 7 antioxidant and 6 anti-aging related proteins were identified and their volume was calculated in absolute values and relative volume (%) as shown in Table 2 and Table 3. Among 7 identified antioxidant related proteins, there were 5 proteins up-regulated including GPX2, GCLM, PRDX4, GSTA1 and NQO1, and 2 proteins down-regulated including NF-kB1 and TNF-alpha. GPX2, an antioxidant enzyme, is one of two iso-enzymes being responsible for the majority of the glutathione-dependent hydrogen peroxide-reducing activity in the epithelium of the gastrointestinal tract [8, 9]. PRDX4 is also an antioxidant enzyme which reduces hydrogen peroxide and alkyl hydroperoxides to water and alcohol with the use of reducing equivalents derived from thiol-containing donor molecules. Meanwhile GCLM has the function of protein binding, protein heterodimerization activity, glutamate-cysteine ligase activity, oxidoreductase activity and glutamate-cysteine ligase catalytic subunit binding [8, 10]. GSTA1 conjugates reduced glutathione to a wide number of

Table 2. List of identified anti-oxidant proteins of control and treated NF.

Protein name	**NCBI accession No. [9]**	**Theoretical [9]**		**Absolute spot volume**		**Relative spot volume (%)**	
		pI	**MW (kDa)**	**Control**	**Treated**	**Control**	**Treated**
GPX2	P18283	7.64	21.803	649696	799440	100	123.05
GCLM	P48507	5.7	30.727	109527	178459	100	162.94
PRDX4	Q13162	5.86	30.539	642111	772519	100	120.31
GSTA1	P08263	8.91	25.631	504105	1639701	100	325.27
NQO1	P15559	8.91	30.867	143748	667405	100	464.29
NFKB1	P19838	5.2	105.356	89552	57892	100	64.65
TNF-α	P01375	6.44	25.644	795550	781834	100	98.28

exogenous and endogenous hydrophobic electrophiles. And NQO1 prevents the one electron reduction of quinones that results in the production of radical species [10, 11]. They are natural anti-oxidants of the skin which help skin protect itself from free radical damage [12]. Noticeable, GSTA1 was up-regulated more than 3 times and NQO1 was up-regulated nearly 5 times.

It was reported that free radicals up-regulate nuclear transcription factor-kappa B (NF-kB1) which response for up-regulating the transcription of tumor necrosis factor alpha (TNF-alpha) [12]. However in treated NF, the expression of NF-kB1 was down-regulated and lead to the decrease of TNF-alpha amount. This proved that *Coffea robusta* extract containing anti-oxidative compounds which destroyed or inhibited free radicals. Therefore, the skin treated with *Coffea robusta* extract could be prevented from intracellular structure damage including DNA.

Table 3. List of identified cellular senescence related proteins of control and treated NF.

Protein name	**NCBI accession No. [9]**	**Theoretical [9]**		**Absolute spot volume**		**Relative spot volume (%)**	
		pI	**MW (kDa)**	**Control**	**Treated**	**Control**	**Treated**
CELA2B	P08218	6.8	28.809	487268	476823	100	97.86
CELA3A	P09093	6.3	29.488	230524	200008	100	86.76
CELA3B	P08861	5.85	29.263	72469	33412	100	46.11
MMP3	P08254	5.77	53.977	256916	130247	100	50.70

In collagen synthesis, there was no related protein which was identified. However, three proteins in elastase family were identified which were CELA2B, CELA3A and CELA3B. Elastase is an enzyme family which breaks down elastin, an elastic fiber that, together with collagen, determines the mechanical properties of connective tissue [10, 11]. The three elastase amounts in treated NF were all down-regulated. This meant the elastin degradation occurred in NF cells treated by Coffea robusta extract less than in the cells without treating. Therefore skin treated by *Coffea robusta* extract could have higher chance to return to its original position when it is poked or pinched. Furthermore, the expression of MMP3, a degrading enzyme, decreased about 50%.

4. CONCLUSIONS

In conclusion, roasted *Coffea robusta* extract has strong anti-oxidant capacity with IC50 value for DPPH scavenging is 33.2 mg/L. When being dosed on human fibroblast, the extract significantly up-regulate the natural antioxidant enzymes including GPX2, GCLM, PRDX4, GSTA1 and NQO1, as well as destroyed or inhibited free radicals which resulted in down-regulation of NF-kB1 and TNF-alpha.

Roasted *Coffea robusta* extract inhibits the degradation of elastin and dermal extracellular matrix with the down-regulation of 4 proteins including CELA2B, CELA3A, CELA3B and MMP3.

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TÓM TẮT

ĐÁNH GIÁ TÁC DỤNG CHỐNG OXI HÓA VÀ CHỐNG LÃO HÓA TRÊN NGUYÊN BÀO SỢI NGƯỜI (HUMAN FIBROBLAST) CỦA DỊCH CHIẾT TỪ *COFFEA ROBUSTA*

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Dịch chiết từ *Coffea robusta* được đánh giá hoạt tính chống oxi hóa và chống lão hóa trên nguyên bào sợi người. Hạt cà phê *Coffea robusta* thu hoạch tại thành phố Buôn Ma Thuột được rang và chiết bằng nước nóng. Hoạt tính chống oxi hóa của dịch chiết được xác định thông qua khả năng kháng gốc tự do DPPH. Đồng thời dịch chiết được thử trên nguyên bào sợi người và phân tích điện di 2 chiều được thực hiện để ghi nhận sự thay đổi của các protein có liên quan đến quá trình lão hóa. Kết quả cho thấy dịch chiết từ *Coffea robusta* là một tác nhân chống oxi hóa mạnh với khả năng kháng gốc tự do DPPH có giá trị IC50 thấp (33,2 mg/L), làm tăng rõ rệt 5 enzyme chống oxi hóa tự nhiên (GPX2, GCLM, PRDX4, GSTA1 và NQO1), cũng như giảm 2 protein hỗ trợ quá trình hình thành gốc tự do (NF-kB1 và TNF-alpha). Hơn hữa, dịch chiết còn là một nhân tố chống lão hóa tiềm năng khi làm giảm 4 protein có liên quan đến sự thoái hóa elastin và ma trận ngoại bào (CELA2B, CELA3A, CELA3B và MMP3) khi thử trên nguyên bào sợi người.

Từ khóa: chống lão hóa, chống oxi hóa, *Coffea robusta* , nguyên bào sợi người.