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PROTECTIVE EFFECT OF PBC 140 BEAN EXTRACT ON THE SUPPRESSION OF MATRIX METALLOPROTEINASE-1 (MMP-1) IN HUMAN DERMAL FIBROBLASTS

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ABSTRACT – *Excess exposure to solar ultraviolet (UV) irradiation that often leads to decreases of procollagen production and subsequent breakdown of collagen fibers with regard to skin photoageing, are often due to overexpression of matrix metalloproteinases (MMPs). Although several evidences have been reported pertaining to cocoa intake in the improvement of skin health, molecular mechanisms responsible for the beneficial effects of PBC 140 cocoa bean extract (CBE) on the inhibition of MMP-1 expression in extracted ribonucleic acid of human dermal fibroblasts, adult (HDFa), have not yet thoroughly investigated. In this study, reduction of MMP-1 expression of the CBE at $5x10^2$ and $1x10^3$ μg/mL, by 9.34- and 25-folds relative to the calibrator, respectively, have verified its significant ($p<0.05$) photoprotective effect for skin anti-ageing. The results demonstrate that CBE of PBC 140 inhibits MMP-1 expression, suggesting that the extract could be potentially used in the prevention of skin ageing through MMP-1 inhibition, thus dermal matrix can be protected that leads to overall reduction in wrinkle formation. From these findings, further researches have been proposed in the development of Malaysian PBC 140 bean extract as a potential active material for cosmeceutical as well as nutraceutical benefits due to the encouraging results in the suppression of MMP-1.*

Keywords: PBC 140, CBE, HDFa, MMP-1, skin photoageing

INTRODUCTION

Skin photoageing (extrinsic ageing) is primarily induced by an exposure of skin to solar radiation, particularly to the ultraviolet (UV) part of the spectrum (Kim *et al.*, 2004). The detrimental effects of UV irradiation on skin are mediated by the increased formation of free radicals and by the direct absorption of energy by cell biomolecules. The free radicals may directly alter the structure of DNA (Ouhtit and Ananthaswamy, 2001; Laga and Murphy, 2009), proteins (Laga and Murphy, 2009), lipids (e.g. lipid peroxidation) (Sorg *et al.*, 2007) and modulate the gene expression of a wide range of proteins through modulation of various signal transduction pathways. These events are connected with changes in the composition and turnover of the extracellular matrix (ECM), which characterize skin ageing.

Reduction in the main components of ECM due to natural skin chronoageing and photoageing, may arise from their reduced synthesis and increased degradation due to an elevation of MMP expression (Chung *et al.*, 2001; Fisher *et al.*, 2009). MMPs are the family of proteolytic enzymes that specifically degrade collagen, elastin and other proteins. MMP gene expression can be triggered by an elevation of oxidative stress (Kim *et al.*, 2004; Wenk *et al.*, 2004). MMP-1 (collagenase-1) preferentially degrades fibrillar collagens, which maintain the strength of connective tissue. UV irradiation induces the expressions of interstitial MMP-1 both in HDFa *in vitro* and human epidermis *in vivo* (Wenk *et al.*, 2004). The level of MMP-1 tends to be higher in naturally aged skin of old subjects in comparison to the young person (Fisher *et al.*, 2009).

One approach to protect skin from harmful effects of UV irradiation is to use active

photoprotectives. There were several reports regarding natural products to play as a substantial character on the downregulation of MMPs in UV-irradiated cells (Chae *et al.*, 2011; Zhang *et al.*, 2009). The highly efficient ones are phenolics-containing plant seeds/beans which were proven in the inhibition of MMPs, therefore boosting synthesis of collagen and elastin (Varma *et al.*, 2017; Chiang *et al.*, 2011). *Theobroma cacao* bean which is classified under the genus *Theobroma* and belongs to the *Sterculiaceae* family, has been blessed with major bioactive components such as epicatechin, catechin, and methylxanthines (Scapagnini *et al.*, 2014). In Malaysia, potential cocoa clone with premium quality, namely PBC 140, has been developed in Malaysian Cocoa Board research and development stations. Although evidences pertaining to cocoa intake in the improvement of skin health were regularly reported, molecular mechanisms responsible for the beneficial effects of PBC 140 cocoa bean extract (CBE) on the inhibition of MMP-1 expression in extracted ribonucleic acid of human dermal fibroblasts, adult (HDFa), have not yet thoroughly investigated. This study aims to investigate overall potential of the PBC 140 clone for its anti-ageing activities in term of photoprotective effect against MMP-1 gene expression in HDFa cells *in vitro*.

MATERIALS AND METHODS

Materials and Chemical Reagents

PBC 140 clone was collected from the Malaysian Cocoa Board, Cocoa Research and Development Centre, Jengka Pahang Malaysia. Fresh beans were collected from cocoa pods without any sign of cocoa pod borer (CPB) infestation, and the beans were manually removed without any typical subsequent processes, i.e., drying, fermentation and roasting, in order to preserve the valuable functional components. Chemical reagents and other materials were obtained from the following commercial sources: Bio-Diagnostics Sdn. Bhd. (GIBCO, catalogue number C-013-5C; Lot number 1378119) was the sole supplier of HDFa, Dulbecco's modified Eagle's medium (DMEM) and Trypsin (Trypsin LE™). Fetal bovine serum (FBS) and Streptomycin/ Penicillin (antibiotics) were purchased from Biowest whereas Phosphate Buffer Saline (PBS, 10X) was supplied by R&M Chemicals, UK.

Methods

Cell Culture

Initially, the cryopreserved HDFa of $\geq 5x10^5$ viable cells/1 ml/vial were grown in a 25 cm^2 tissue culture flask containing a mixture of culture medium consisting of 445 mL DMEM, 50 mL 10% FBS and 5 mL 1% antibiotics. The cells were incubated at 37°C in 5% CO_2 and 95% humidified incubator (MEMMERT GmbH, Schwabach Germany). During this process the cells adhered to the culture flask. The growth media was refreshed for every two days until at least 80% confluence ($1x10^4$ cells/cm^2), and later trypsinized with Trypsin LE™ where the subcultured cells were plated in new flasks at ≅ $1.3x10^4$ cells/cm^2 density by using the same medium (Ingrid *et al.*, 2012). The cells were seeded into each well of 96-well plates ($1x10^4$ cells/cm^2) and cultured with 250 µL supplemented DMEM at 37°C with 5% CO_2. After 24 hrs, cells cultured in serum free medium were treated with the CBE ranging from $2.5x10^2$ to $1.0x10^3$ µg/mL.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR)

Ribonucleic acid (RNA) extracted from the untreated (control) as well as HDFa cells that were subjected to the CBE treatment for 24 hrs by an RNeasy® Mini Kit (QIAGEN, Saint Louis, USA), were according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and analyzed using the QuantiTech SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA). For real time PCR analysis, the cDNA samples were run in triplicate and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Briefly, the reaction was carried out at 95°C for 15 min, followed by 48 cycles of amplification (94°C for 15 s, and 60°C for 60 s). The PCR efficiency (E) was calculated as follows: $E(\%) = (10^{-1/slope} - 1) \times 100$. Acceptable E value was described as between 90 and 110% (Kubista *et al.*, 2006). The comparative threshold cycle (C_T) method was used to analyze the data obtained by real time PCR (Giulietti *et al.*, 2001). Specific primer pairs (Genotech, Daejeon, Korea) were as

follows: MMP-1 forward 5′–GGA GGG GAT GCT CAT TTT GAT G–3′, MMP-1 reverse 5′–TAG GGA AGC CAA AGG AGC TGT–3′, MMP1' forward 5′–AGC TAG CTC AGG ATG ACA TTG ATG–3′, MMP1' reverse 5′– GCC GAT GGG CTG GAC AG–3′, GAPDH' forward 5′–CGA CAG TCA GCC GCA TCT T–3′ and GAPDH' reverse 5′–CCC CAT GGT GTC TGA GCG–3′.

Statistical Analysis

All plotted data were expressed as the mean ± SD. For gene array/mRNA expression data analysis, the unpaired t-test was used to detect significant changes in gene expression between the treatment and negative control (calibrator) (p<0.05) (Minitab version 14). Inhibition of gene expression was detected in which sample measured through relative expression to the calibrator has significantly lower copy numbers (p<0.05) (Lephart, 2013).

RESULTS AND DISCUSSION

CBE of PBC 140 Prevents MMP-1 Upregulation in HDFa

The effectiveness of natural products on MMPs downregulation in UV-irradidated cells have been reported in many studies (Pittayapruek *et al.*, 2016). Degradation of collagen as a consequence of MMPs upregulation in extrinsic damaged skin has often occurred in an environment filled with ROS accumulation. Thus, MMPs inhibition has been seen as a compatible method for the evaluation of skin anti-ageing. To the best of our knowledge, this study is the first attempt to clarify the possibility that CBE of PBC 140 may be considered as a possible wrinkle-reducing candidate for topical application. Therefore, there is a need to elucidate the effect of PBC 140 on MMP-1 suppression in HDFa cell lines.

According to Patwardhan and Bhatt (2015), the HDFa cell lines have been used in the evaluation of many plant flavonoids protective effects against damage caused by UV light radiation in humans. In this study, an assessment has been conducted on the MMP-1 level using real time quantity polymerase chain reaction (RT-qPCR) in HDFa cell lines treated with CBE in a dose-dependent manner (2.5 x10^2 to 1.0x10^3 µg/mL) (Figure 1A) whereas GAPDH was used as an internal control (Figure 1B). Cytotoxic effect which was earlier conducted in HDFa cell line for 24 hrs at 39.1–1.0x10^4 µg/mL, has indicated that concentration was cytocompatible up to 1093.75 µg/mL where any MMP-1 inhibition observed was not influenced by any cytotoxic effect at the concentrations below the IC_{50} value. Therefore, 1.0x10^3 µg/mL has been chosen as an optimal dose or the highest CBE concentration permitted for the effect of PBC 140 on MMP-1 suppression in HDFa cell lines. The MMP-1 level was significantly reduced by the CBE treatment at 1.0x10^3 µg/mL compared to control as well as the other two concentrations (2.5x10^2 and 5.0x10^2 µg/mL).

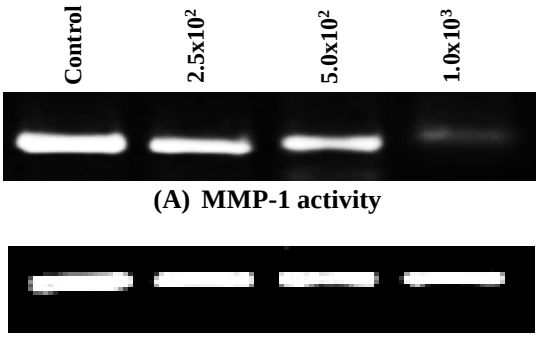

Figure 1: Polyacrylamide gel electrophoresis analysis of the qPCR products. (A) MMP-1 mRNA expression in HDFa cell lines which were treated with the CBE (2.5 x10^2 to 1.0x10^3 µg/mL, and (B) GAPDH was used as an internal control

The equations of the linear regressions used to indicate the PCR efficiency pertaining to the copy number of MMP-1 are given in Table 1. A 10-fold dilution series was created from a sample with a known concentration of nucleic acid of HDFa, resulting in a set of standard containing 10^{-1}–10^{-7} copies of template. A curve with two regression lines was constructed, with the logarithm of the initial copy number of optimal dose or the highest CBE concentration that would not induce cytotoxic effect (HD; 1.0x10^3 µg/mL) and its lower dose (LD; 5x10^2 µg/mL), were plotted along the x-axis and their respective cycle threshold (C_T) values plotted along the y-axis, indicating 108.21% and 92.04%

efficiency for HD and LD, respectively (Table 1). These equations, which were taken into account the various successive steps in the whole procedure, indicated the reliability and reproducibility of RT-qPCR quantification method.

Table 1: PCR efficiency showed from the regression lines of LD and HD copy number of MMP-1

Sample	***Regression Lines***	R^2	***PCR efficiency, E*** $[E(\%) = (10^{-1/slope} - 1) \times 100]$
LD ($5x10^2$ μg/mL)	$Y = -3.5286x + 28.4079$	0.9989	92.04%
HD ($1.0x10^3$ μg/mL)	$Y = -3.1390x + 35.9975$	0.9996	108.21%

Note: HD: High Dose
LD: Low Dose

Kim *et al.* (2016) have showed that *in vivo* ethanolic cocoa extract treatment on mice has elicited an inhibition of MMP-1 protein expression in a concentration-dependent manner where the mRNA levels of UVB-induced MMP-1 was significantly suppressed. In addition, formation of wrinkle was significantly reduced upon ingestion of 4 g/day of cocoa extract for 24 weeks in a clinical study conducted by Kim *et al.* (2016).

In the present study, relative quantification of MMP-1 to the calibrator is exhibited in Table 2. The CBE treatment of LD and HD for 24 hrs could cause about 9.34- and 25-folds downregulation of UV-induced MMP-1 activity in a dose-dependent manner. Their relative expression to the calibrator which have been determined by using C_T values, were significantly different (p<0.05) (Figure 2). According to Al-Olayan *et al.* (2014), significant decreased of glutathione peroxidase and glutathione reductase have been confirmed by the decreasing in mRNA expressions by 2.9-fold and 4-fold, respectively, after 12-weeks of *in vivo* liver tissue treatment with quercetin and kaempferol-rich Cape gooseberry (*Physalis perruviana* L.). Furthermore, treatment of 916.74 μg/mL epigallocatechin-3-gallate (EGCG) which is a major polyphenol present in green tea, has been shown to inhibit the MMP-1 expression by 16-fold (Sen and Chatterjee, 2011). Relative decreasing of MMP-1 expression by CBE sample ($1.0x10^3$ μg/mL) was comparatively higher than the ones showed by the previous findings. The results suggest that the CBE sample may downregulate the expression of UVA-induced MMP-1 and thus inhibiting skin ageing.

Polyphenols have been said to inhibit MMP-1 expression during its constant occurrence during intrinsic and extrinsic oxidative damages, as a consequence to healthy and younger looking skin through collagen and elastin stimulation (Korac and Khambholja, 2011). Some studies have exhibited that polyphenol-rich extracts derived from beans, i.e., *Coffea arabica* (Chiang *et al.*, 2011) and *Terminalia catappa* L. (Wen *et al.*, 2011) were able to inhibit the activation of MMPs by preventing skin cells from UVB-induced photoageing. Stratum corneum which was appeared as a fundamental target of oxidative damage due to its skin barrier structure/function, has been significantly protected against UV-induced expression of MMP-1, MMP-8 and MMP-13 by polyphenol EGCG in *Phaseolus vulgaris* L., strawberry and common walnut (Bae *et al.*, 2008; Schurer and Elias, 1991), indicating significant property of polyphenols in the inhibition of MMPs.

Table 2: Determination of the relative quantification of MMP-1 to the calibrator in LD and HD samples of the CBE

Sample	***Replicate***	***C_T MMP-1 (Target)***	***C_T GAPDH (Reference)***	***Expression $2^{[CT\ (GAPDH)\ -\ CT\ (MMP\text{-}1)]}$***	***Normal Expression (Calibrator/ Calibrator)***	***MMP-1 Expression (MMP-1/Calibrator)***
Normal	1	27.9	27.4	0.707	1	-
(Calibrator)	2	28.2	27.7	0.707	1	-
	3	28.6	27.9	0.616	1	-
Average					1 ± 0.00	-
LD	1	31.6	28.0	0.082	-	0.116
($5x10^2$ µg/mL)	2	32.1	28.3	0.072	-	0.102
	3	32.5	28.5	0.063	-	0.102
Average					-	0.107 ± 0.008
HD	1	33.2	27.7	0.022	-	0.031
($1.0x10^3$ µg/mL)	2	33.4	28.0	0.024	-	0.034
	3	33.1	28.2	0.033	-	0.054
Average					-	0.040 ± 0.013

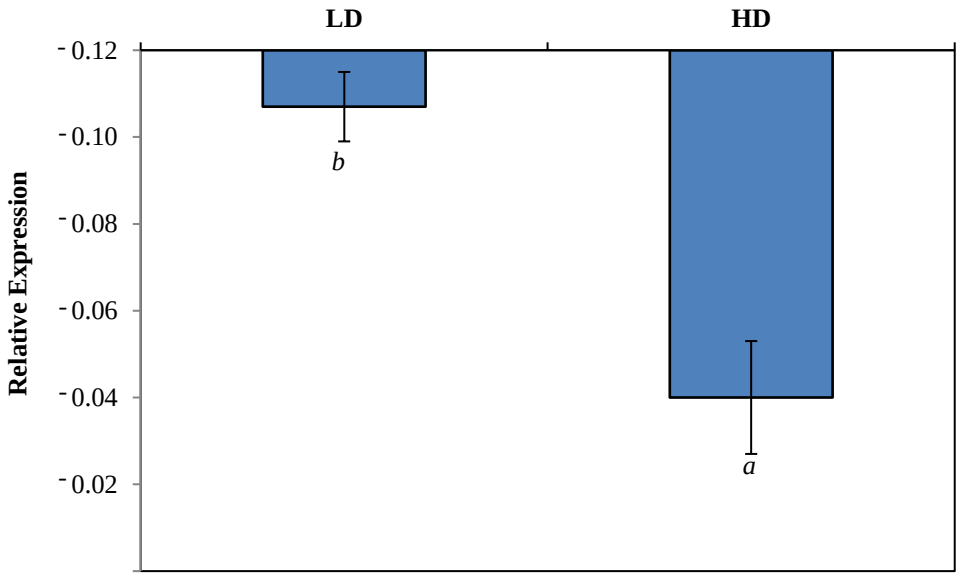

Figure 2: Relative MMP-1 expression of LD and HD samples of the CBE
*(*Means in the horizontal bar followed by different letters were significantly different at p<0.05)*

Polyphenols have also involved in the cross-linking collagen to elastin fibres and the induction of elastin formation within dermis skin layer (Noblesse *et al.*, 2004). The findings of previous researches regarding antioxidant roles were parallel with the present observations, where flavonoids-containing CBE specifically at 1000 µg/mL has significantly reduced the gene expression of MMP-1 by 25-fold relative to the calibrator (Table 2). Thus, the inhibition of MMP-1 gene expression by CBE of PBC 140 in the present study support the antioxidant capacity and *in vitro* anti-ageing activities which is most probably due the presence of its significantly high epicatechin content (133.70±0.64 mg/g DW), earlier on detected through separation of the compound on reversed phase-high performance liquid chromatography/ diode array detector (RP-HPLC/DAD) using an Ascentis C_{18} column (5 µm, 15 cm x 4.6 mm, i.d., Agilent, USA) with water:methanol:acetic acid (87:8:5) as a mobile phase (Norliza, 2019).

CONCLUSION

Reduction of MMP-1 expression of the CBE at $5x10^2$ and $1x10^3$ µg/mL, by 9.34- and 25-folds relative to the calibrator, respectively, have verified the significant anti-wrinkles effect of PBC 140 cocoa bean extract for skin anti-ageing. The results also demonstrate that the CBE inhibits MMP-1 expression through MMP-1 inhibition, thus dermal matrix can be protected that leads to overall reduction in wrinkle formation. From these findings, further researches have been proposed in the development of CBE as an alternative biomaterial for cosmeceutical as well as nutraceutical benefits.

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