**O-FNN-01**

# Antioxidant activity of porcine placenta and its cytotoxic effect on HaCaT cell

**Pantanat Mahasupachai[1*], Praneet Opanasopit[2] , Nisa Patikarnmonthon[1,3]**

*[1]Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
*[2]Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakorn Pathom, 73000, Thailand*
*[3]Mahidol University-Osaka University Collaborative Research Center, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

**Corresponding author. E-mail: Pantanat.m@gmail.com*
**DOI :**

## ABSTRACT

Porcine placenta, previously a low-value waste from pig farm, is now being popular material in supplementary food and cosmetic products. Porcine placenta (PP) is claimed to be beneficial for skin improvement as whitening and anti-wrinkle. This research aims to investigate properties of PP and also its effect on the immortalized human keratinocyte (HaCaT) cells including protein content, protein profiles, antioxidant activity, cytotoxic effect, and ability to promote cell proliferation. The protein content of PP measured by Lowry assay was 27.04 %(w/w). The ABTS assay was used to determine the antioxidant activity of PP. The results suggested that at concentration of 5 mg/ml, PP provides 83.6% of antioxidant activity. The cytotoxic effect of PP was studied on HaCaT cells using MTT assay. The results suggested that PP at a concentration of up to 0.1 mg/ml does not affect the cell viability. In addition, at 0.01 and 0.1 mg/ml, PP tends to promote cell proliferation. These results can be beneficial for further study of bioactivity of porcine placenta in order to apply in pharmaceutical and cosmetic aspects.

**Keywords:** Porcine placenta, Skin improvement, Antioxidant activity, Cytotoxicity, HaCaT.

## INTRODUCTION

Over decades, pork has become the second most popular meat in Thailand. During 2015-2017, pork consumption in Thailand was at 10.4 kg/capita and is projection to be 11.7 kg/capita in 2027, suggesting demand of pork meat in the market (OECD/FAO, 2018). To serve the demand for pork consumption, pig farming has enlarged and become industrialized, this leads to the disposal of large volume of waste from the production, including feces, urine, blood, etc. Pig farm has to minimize the cost and find an appropriate way to utilize those wastes. General option is to transform them into fertilizer or biogas. Another option is to extract the active ingredients from waste and convert them into a high-value product.

Porcine placenta is a type of waste found in pig farrowing farm that contain biologically active compounds including peptides, vitamins, and growth factors (Tiwary et al., 2006). Nowadays, extracts from placenta can be used as an active ingredient in cosmetics and food supplements such as whitening (Yamasaki et al., 2014) and anti-wrinkle (Yoshikawa, 2013). It is also considered as promising source for therapeutic purpose because active compounds from placenta can be used for wound healing, prevent fibrosis and acceleration of epithelization (Hong, Lee, Hahn, Kim, & Lew, 2010). In previous research, the antioxidant compound was found in porcine placenta extract, with ability to prevent oxidative stress from $H_2O_2$ and promote cell proliferation (Choi et al., 2014).

The aim of this research is to investigate the activity as well as effect of porcine placenta on the cell in order to convert the raw porcine placenta from pig farm into value-added product which can be further developed into cosmetic or pharmaceutical applications.

## MATERIAL AND METHODS

### Materials

Raw porcine placenta used in this study was provided by Betagro Co. Ltd., Thailand as frozen sample. HaCaT cells were obtained from collaborator (Assoc. Prof. Dr. Tavan Janvilisri). Potassium sodium tartrate, sodium carbonate, and ABTS was purchased from Sigma Aldrich, USA. Copper sulphate and sodium hydroxide were obtained from Merck, Germany. NuPAGE™ 4-12% Bis-Tris Protein Gels was purchased from Invitrogen, California.

**Preparation of homogenized PP**

Porcine placenta was prepared according to the previous report (Hye Yeon Choi et al., 2014), frozen porcine placenta was thawed and washed in 0.9% w/v NaCl at 4°C to remove all traces blood. After that, the sample was soaked in phosphate buffer saline solution (containing 0.137 M NaCl, 0.0027 M KCl, 0.01 M $Na_2HPO_4$, and 0.0018 M $KH_2PO_4$) and transferred to the blender for mixing. The blended sample was stored at 4°C prior to lyophilizing step.

**Determination of protein content in porcine placenta**

The total protein content of porcine placenta was measured by Lowry assay, according to the previous described protocol (Lowry, Rosebrough, Farr, & Randall, 1951). Complex-forming reagent A (10 mg/ml Potassium sodium tartrate and 5 mg/ml $CuSO_4$ in distilled water) and reagent B (4 mg/ml NaOH and 20 mg/ml $Na_2CO_3$ in distilled water) were mixed at ratio of 1:50 (v/v). After that, 2.5 ml of mixed reagent was added into sample, the reaction took place for 10 minutes at 37°C, allowing the formation of protein complex $Cu^+$. Then 250 µl of Folin reagent was added into the mixture following by incubating at 37°C for 30 minutes. Finally, total protein concentration was determined by measuring the absorbance of each mixture at wavelength of 750 nm using spectrophotometry.

**SDS-PAGE**

The protein was separated based on the molecular mass using SDS-PAGE. Ten microgram of total protein from PP was electrophoresed on NuPAGE 4-12% Bis-Tris Protein Gel. The molecular weight of PP was determined using PageRuler™ Plus Prestained Protein Ladder. After electrophoresis, separated proteins were stained with 0.25% Coomassie blue (Bio-Rad) dissolved in 25% methanol and 10% (v/v) acetic acid for 30 minutes and de-stained in alkaline phosphatase buffer (100 mM NaCl, 5mM $MgCl_2$, 100mM Tris pH 9.5). The gel was visualized under transilluminator for observing the protein profile. The estimated molecular weight of proteins in PP were analyzed by using ImageJ v1.52a software.

**Antioxidant activity**

The antioxidant activity of PP was performed based on calorimetric assay according to previous described method (Van den Berg et al., 1999). Briefly, PP was dissolved in deionized water at desirable concentration. 10 µl of each sample was mixed with 200 µl of ABTS solution. The reaction mixture

was kept in the dark for 10 minutes at room temperature. PP sample solution was replaced with deionized water in the reaction mixture to be used as a control. The absorbance of each sample was observed by spectrophotometry at wavelength of 734 nm. The ABTS radical scavenging activity was calculated by the following equation.

% ABTS = [(Abs. of control – Abs. of sample) / Abs. of control] x 100

Where Abs. control is the absorbance of the control and Abs. sample is the absorbance of the ABTS in each sample.

**Cell culture**

In this study, immortalized human keratinocyte (HaCaT) cell was used as model cell to study the effect of PP on cell viability and cell proliferation. HaCaT cells were grown in DMEM containing 4.5 g/l glucose with L-glutamine and sodium bicarbonate supplemented with 10% of fetal bovine serum (FBS), 1% (v/v) of penicillin-streptomycin, and 1% (v/v) of sodium pyruvate. HaCaT cells were incubated at 37°C with 5% $CO_2$. Complete medium was changed every other days, the cells were passaged at 80% confluence.

**Cytotoxic effect of PP on HaCaT.**

HaCaT cells were cultured in 96-well plate treatment at seeding density of $2x10^4$ cells/well and incubated at 37°C with 5% $CO_2$ for 6 hours prior to treatment. Afterward, the cells were treated with different concentrations of PP (ranging from 0.001-5 mg/ml) for 24 hours. Cell with culture medium and 10% DMSO were used as untreated control and positive control, respectively. After that, MTT assay was used to determine the cell viability.

**Effect of PP on cell proliferation.**

HaCaT cells were seeded into 96-well plate at seeding of $1x10^4$ cells/well and incubated at 37°C with 5% $CO_2$ for 6 hours prior to treatment. Then HaCaT cells were treated with sublethal concentration (ranging from 0.001-0.1 mg/ml) for 6 hours before MTT assay. The cell viability was observed and compared with untreated cell.

**MTT assay**

The MTT assay was used to study the metabolic activity of the cell which corresponded to the cell viability. The method used in this study was modified according to the previous report ( Mosmann, T. ,1983) . After treatment, culture media in each well was removed and the cells were washed with PBS. Then, 100 µl of 0.5 g/ml MTT solution was added into each well. The cells were then incubated in the dark at 37°C supplied with 5% $CO_2$ for 30 minutes. The solution in each well was replaced with 100 µl of acidified isopropanol to dissolve formazan products. Afterward, the samples were analyzed by using microplate spectrophotometer ( MultiskanTM GO Microplate, Thermofisher Life Science) to observe the absorbance at wavelength of 570 nm for metabolic signal and reference wavelength at 630 nm for substrate interference.

**Statistical analysis**

All data were analyzed using Statistical Package for the Social Sciences (SPSS) 18.0 software, release 2009 (SPSS Inc., Headquarters, Chicago, IL) for statistical comparison. Data was reported as mean, standard deviation.

## RESULTS

**Protein content of homogenized porcine placenta**

Total protein concentration of porcine placenta was measured by the Lowry assay (Lowry et al., 1951). In this study, bovine serum albumin (BSA) at different concentration ranging from 0- 240 µg/ml was used to plot the standard curve for estimating the protein content in the sample as shown in Figure 1. The estimated protein concentration of PP was 27.04%(w/w).

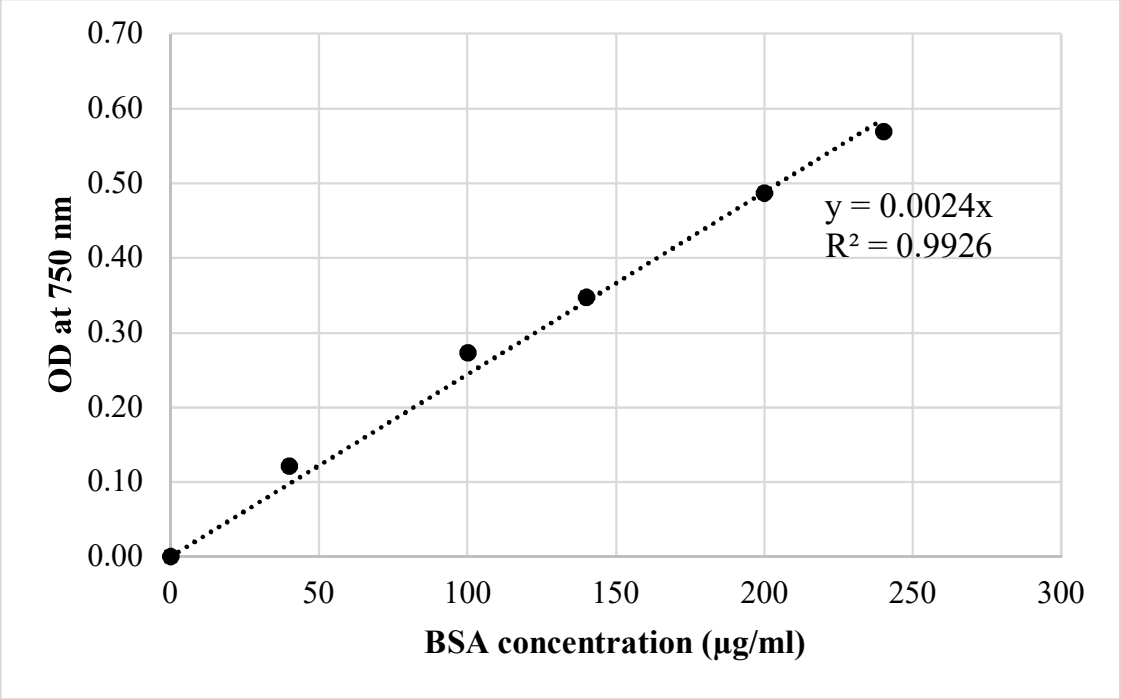


**Figure 1.** The standard curve for protein concentration, using BSA as reference protein.

**SDS-PAGE**

In this study, SDS-PAGE was used to observe the protein pattern in PP. The result shown in Figure 2 suggested that PP contains various sizes of protein with estimated size of about 15 kDa, 44 kDa, 62 kDa, 70 kDa, and 124 kDa. According to the previous report, the 70 kDa is correlated to the size of Alpha-feto protein (AFP), the 68 kDa glycoprotein mostly found during fetal development which considered as the protein with antioxidant effect in porcine placenta (Choi et al., 2014). In addition, the 44 kDa protein might be the vascular endothelial growth factor (VGF) (Vonnahme et al. 2001), a glycoprotein with 45 kDa responsible for placental development and related to the fetal growth such as endothelial cell proliferation, migration and capillary permeability (Neufeld et al. 1999; Stacker & Achen, 1999). Protein with molecular weight of around 124 kDa was also observed in raw porcine placenta, with unknown function (Kyung-Hun Jung et al., 2014).

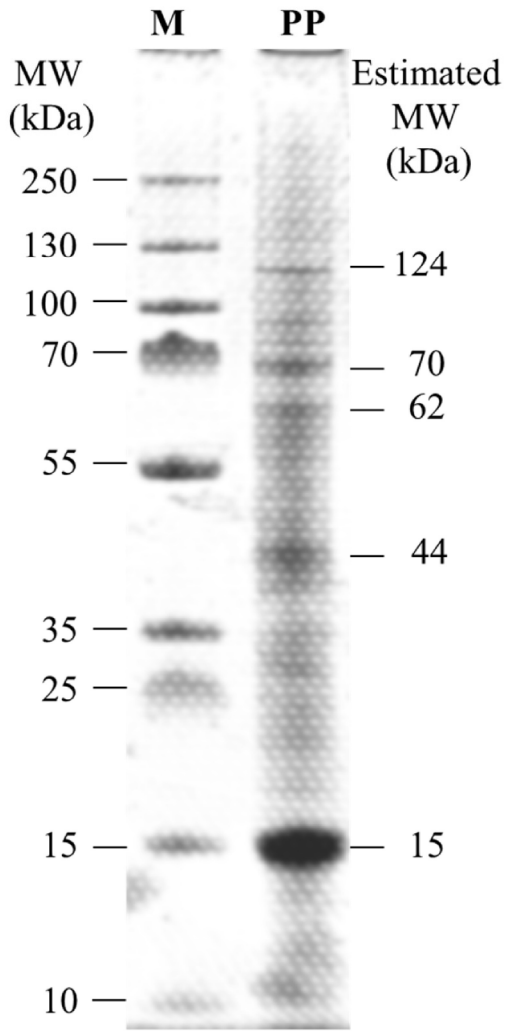


**Figure 2.** SDS-PAGE analysis of porcine placenta. The protein molecular weight was visualized by NuPAGE 4-12% Bis-Tris Protein Gel where M stands for PageRuler™ Plus Prestained Protein Ladder and PP stands for porcine placenta.

**Antioxidant activity of PPE**

The ABTS assay was performed to observe the antioxidant activity of PP, as shown in Figure 3. The result showed that PP provide antioxidant activity at dose dependent manner. The antioxidant activity of PP was 56.5% at 1 mg/ml and up to 83.6% at 5 mg/ml, which was comparable to ascorbic acid at 0.2 mg/ml (89.3%).

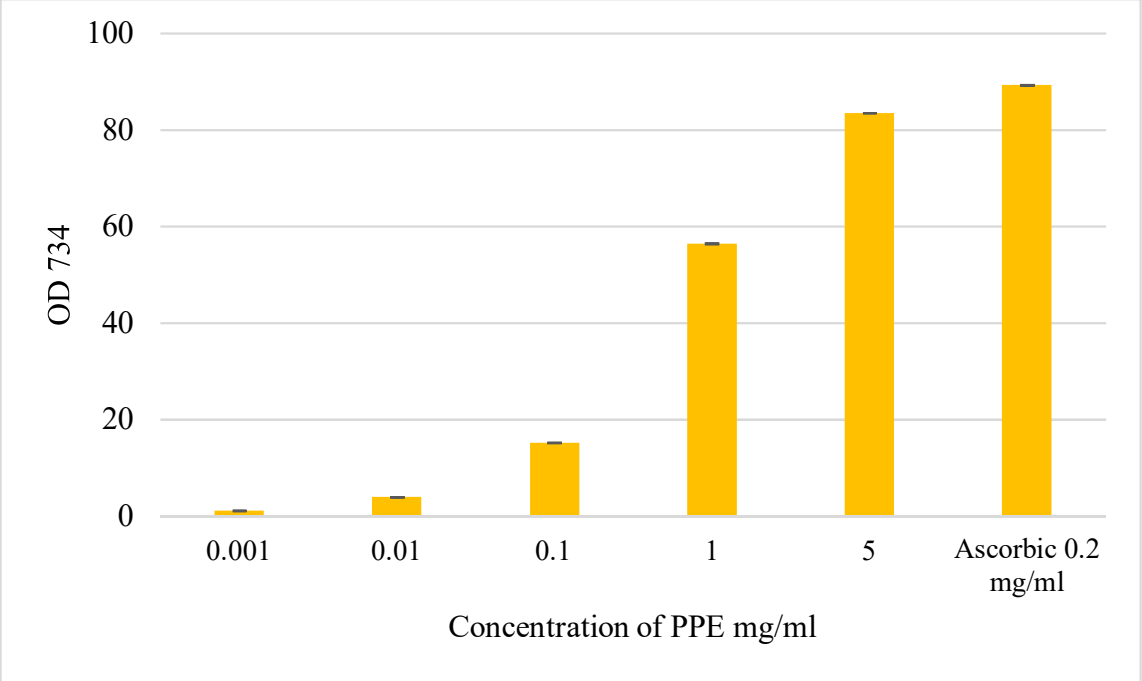


**Figure 3.** The antioxidant activity of 0.001, 0.01, 0.1, 1, and 5 mg/ml of PP and 0.2 mg/ml of ascorbic acid based on ABTS assay. Data are shown as the mean ± standard deviation with n = 3.

**Cytotoxic effect of PP on HaCaT cells**

After 24 hours treatment, the viability of HaCaT cells treated with different PP concentrations were observed. The result in Figure 4 showed that the viability of cells treated with PP at and 5 mg/ml were statistically decreased, suggesting that PP might be toxic to the HaCaT at such concentration. However, the viability of cells treated with lower concentration of PP were high, suggesting that PP was not toxic to HaCaT at concentration up to 0.1 mg/ml. It is interesting that at 0.1 and 0.01 mg/ml of PP, the cell viability was significantly higher than that of control experiment, suggesting the ability of PP to promote cell growth.

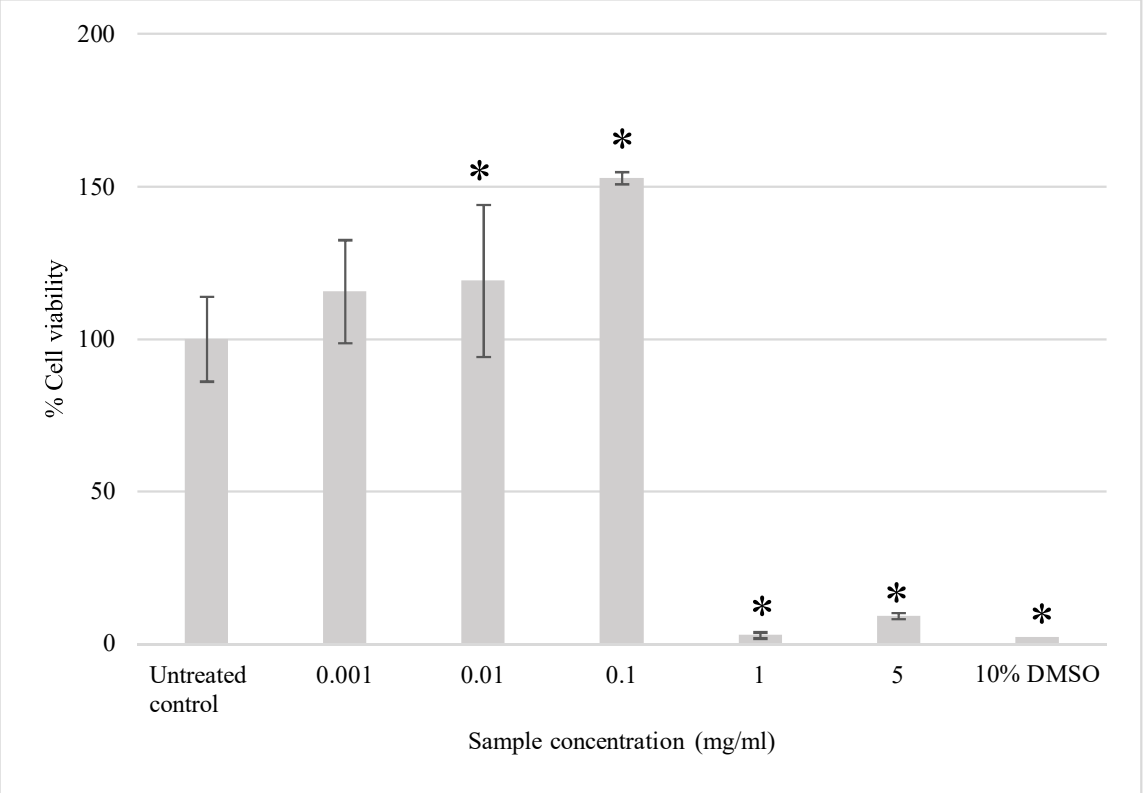


**Figure 4.** Effect of PP on cell viability of HaCaT. Cells were treated with PP at concentration of 0.001, 0.01, 0.1, 1, and 5 mg/ml for 24 hours. The data were compared with untreated control. Cell treated with 10%DMSO was used as positive control in this experiment. Data were shown as the mean ± standard deviation with n = 3, the asterisk (*) represents the statistical difference at p-value of 0.05.

**Effect of PP on cell proliferation**

The effect of PP on cell proliferation was determined using similar assay to that of the cytotoxicity test, but after 6 hours of treatment and at sublethal concentration. The result presented in Figure 5 showed that PP significantly promote growth of HaCaT cells when compared with the untreated control. The cell viability of HaCaT cells incubated with 0.001 and 0.01 mg/ml of PP were reached to 175% and 177%, respectively.

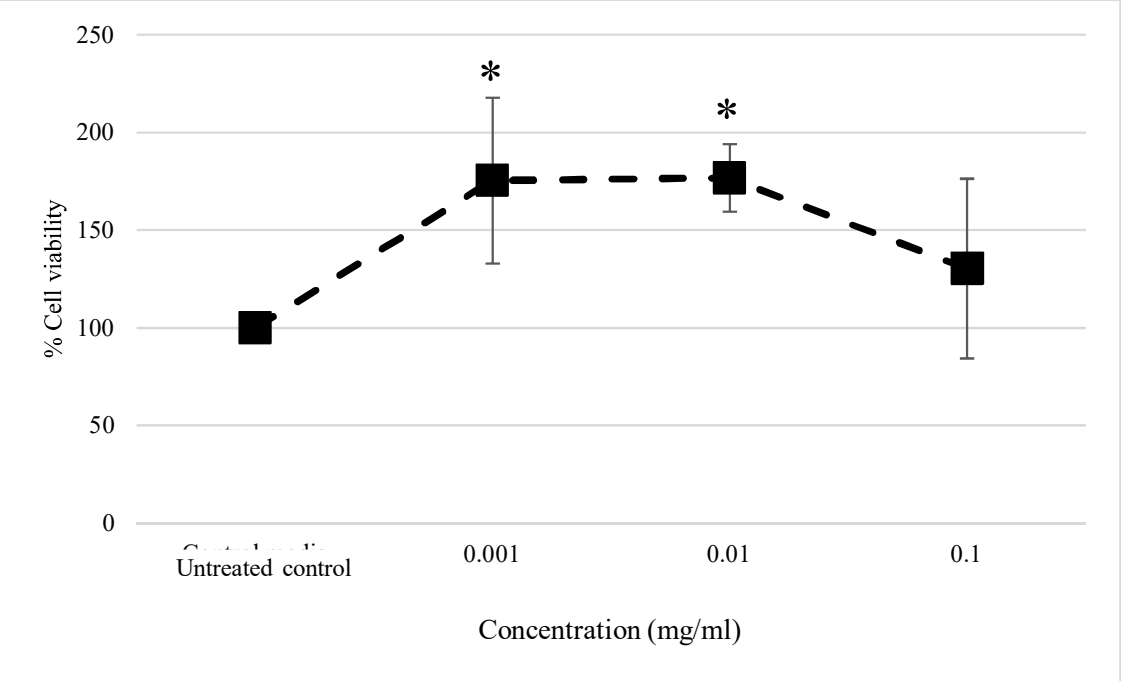


**Figure 5.** Effect of PP on cell proliferation of HaCaT. The cells were treated with PP concentration of 0.001, 0.01, and 0.1 mg/ml for 6 hours. Data were shown as the mean ± standard deviation with n = 3. * = significant level at $p < 0.05$ compared with untreated control.

## DISCUSSION

According to the result, the protein content of PP was at 27% (w/w) which was higher than that of previous work (14.4% (w/w)) (Tang et al., 2013). However, as porcine placenta samples were obtained from different sources, the factors including feed, environment, breed, and also the extraction process of PP might affect the property of sample. The protein profile of PP were correlated with the size of proteins found in previous reports but has to be confirmed by western blotting or ELISA. Interestingly, the majority of protein in PP is with 15 kDa in size, but the information of this protein has not been found on any previous report. In addition, PP showed the antioxidant activity at dose dependent manner, but still be quite low when compared with ascorbic acid, the well-known antioxidant. However, the antioxidant activity of PP was correlated to the previous study (Lee et al., 2016), porcine placenta hydrolysate showed ability to prevent the ROS production on MC3T3-E1 osteoblastic cells by dose dependent. PP at up to 0.1 mg/ml was not affected the cell viability of

HaCaT cells which can be confirmed by another study where up to 100 µg/ml of porcine placenta extract showed no effect on osteoblastic cells (Lee et al., 2016). In addition, PP seems to enhance cell proliferation at low concentration (0.1 and 0.01 mg/ml). Similar result has been reported in the previous study (Imamura et al.,2017), which the porcine placenta extract at concentration of 6.25-100 µg/ml showed ability to promote the proliferation on Normal Human Dermal Fibroblast (NHDF) cells. However, other technique such as BrdU assay might be better option than MTT assay for confirming the effect of PP on cell proliferation.

## CONCLUSION

In this research, some properties of PP were investigated including protein content, SDS-PAGE, antioxidant activity, effect of porcine placenta on cytotoxicity and cell proliferation. The result suggested that PP is rich in protein content and also provides antioxidant protein and antioxidant activity, even in non-purified form. In addition, PP in this work at up to 0.1 mg/ml had no effect on cell viability and tended to promote the cell proliferation on HaCaT cells. This work provides only preliminary step for utilizing of porcine placenta. Additional studies on its bioactivities such as anti- aging, anti-tyrosinase activity, *in vitro* antioxidant activity have to be performed. Suitable extraction method has to be investigated to obtain the active component which rich in such activities. Both extraction technique and bioactivity of porcine placenta can be useful information for developing the porcine placenta extract and converting them to high value product in the future.

## ACKNOWLEDGEMENTS

This work was funded by National Research Council of Thailand and Mahidol University, with partially supported by Faculty of Science, Mahidol University, Central Instrument Facility at Faculty of Science, Mahidol University.

## REFERENCES

Blois, M. S. (1958). Antioxidant Determinations by the Use of a Stable Free Radical. *Nature, 181*, 1199.

Boncler, M., Różalski, M., Krajewska, U., Podsędek, A., & Watala, C. (2014). Comparison of PrestoBlue and MTT assays of cellular viability in the

assessment of anti-proliferative effects of plant extracts on human endothelial cells. *Journal of Pharmacological and Toxicological Methods, 69*, 9-16.

Choi, H. Y., Kim, S. W., Kim, B., Lee, H. N., Kim, S.-J., Song, M., Cho, S.-G. (2014). Alpha-Fetoprotein, Identified as a Novel Marker for the Antioxidant Effect of Placental Extract, Exhibits Synergistic Antioxidant Activity in the Presence of Estradiol. *PLOS ONE*, 9-16.

Hong, J. W., Lee, W. J., Hahn, S. B., Kim, B. J., & Lew, D. H. (2010). The Effect of Human Placenta Extract in a Wound Healing Model. *Annals of Plastic Surgery, 65*, 96-100.

Imamura, Y., Honda, Y., Masuno, K., Nakamura, H., & Wang, P.-L. (2017). Effects of Placental Extract on Cell Proliferation, Type I Collagen Production, and ALP Secretion in Human Osteosarcoma Cell Line Saos-2. *Journal of Hard Tissue Biology, 26*, 157-160.

Jung, K. H., Choi, Y. C., Chun, J. Y., Min, S. G., & Hong, G. P. (2014). Effects of Concentration and Reaction Time of Trypsin, Pepsin, and Chymotrypsin on the Hydrolysis Efficiency of Porcine Placenta. *Korean journal for food science of animal resources, 34(2)*, 151–157.

Lee HY., Chae HJ., Park SY., Kim JH. (2016). Porcine placenta hydrolysates enhance osteoblast differentiation through their antioxidant activity and effects on ER stress. *BMC Complement Altern Med, 16*, 291.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265-275.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods. 65*, 55-63.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. (1999).Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*, *13*,9–22.

OECD/FAO (2018), *OECD-FAO Agricultural Outlook 2018-2027,* OECD Publishing, Paris/FAO,Rome, https://doi.org/10.1787/agr_outlook-2018-en.

Seo, M.-D., Kang, T. J., Lee, C. H., Lee, A.-Y., & Noh, M. (2012). HaCaT Keratinocytes and Primary Epidermal Keratinocytes Have Different Transcriptional Profiles of Cornified Envelope-Associated Genes to T Helper Cell Cytokines. *Biomolecules & Therapeutics, 20*, 171-176.

Shahidi, F., & Zhong, Y. (2015). Measurement of antioxidant activity. *Journal of Functional Foods, 18,* 757-781.

Stacker SA, Achen MG.(1999). The vascular endothelial growth factor family: signalling for vascular development. *Growth Factors,17,*1–11.

Stockert, J. C., Blázquez- Castro, A., Cañete, M., Horobin, R. W., & Villanueva, Á. (2012). MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochemica. 114*, 785-796.

Stoddart, M. J. (2011). Cell Viability Assays: Introduction. In M. J. Stoddart (Ed.), *Mammalian Cell Viability: Methods and Protocols*. 1-6.

Tang, W.-L., Zhang, M., Adhikari, B., & Mujumdar, A. S. (2013). Effects of Preparation and Drying Methods on the Antioxidant Activity of Enzymatically Hydrolyzed Porcine Placenta Hydrolysates. *Drying Technology, 31*, 1600-1610.

Thanapongtharm, W., Linard, C., Chinson, P., Kasemsuwan, S., Visser, M., Gaughan, A. E., Gilbert, M. (2016). Spatial analysis and characteristics of pig farming in Thailand. *BMC Veterinary Research, 12*, 218.

Tiwary, S.K., Shukla, D., Tripathi, A.K., Agrawal, S.N., Singh, M.K., & Shukla, V.K. (2006). Effect of placental-extract gel and cream on non-healing wounds. *Journal of wound care, 15*, 325-328.

Van den Berg, R., Haenen, G.R., van den Berg, H., Bast, A., (1999). Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chemistry 66*, 511–517.

Vonnahme KA, Wilson ME, Ford SP. (2001). Relationship between placental vascular endothelial growth factor expression and placental/ endometrial vascularity in the pig. *Biol Reprod.* 2001;64:1821–1825.

Wu, C. H., Chang, G. Y., Chang, W. C., Hsu, C. T., & Chen, R. S. (2003). Wound healing effects of porcine placental extracts on rats with thermal injury. *British Journal of Dermatology, 148(2)*, 236-245.

Yamasaki, M., Hasegawa, S., Takahashi, H., Kobayashi, Y., Sakai, C., Ashizawa, Y., Fukui, T. (2014). Placental extracts induce the expression of antioxidant enzyme genes and suppress melanogenesis in B16 melanoma cells (Vol. 29).

Yoshikawa, C. (2013). Effect of Porcine Placental Extract on Collagen Production in Human Skin Fibroblasts In Vitro (Vol. 03).