

## Effects of Pterostilbene on Activities and Protein Expression of Cytochrome P450 1A1 (CYP1A1) and Glutathione S-Transferase (GST) in Benzo[a]pyrene-Induced HT-29 Colorectal Cancer Cell Line

(Kesan Pterostilbene terhadap Aktiviti dan Pengekspresan Protein Sitokrom P450 1A1 (CYP1A1) dan Glutathione S-transferase (GST) Pada Sel Kanser Kolorektal HT-29 Teraruh Benzo[a]pirena)

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### ABSTRACT

*Drug Metabolizing Enzyme (DME) has been a target of natural chemopreventive agents to inhibit, retard and reverse the process of carcinogenesis. Pterostilbene, an analog to resveratrol has been reported to possess various pharmacological benefits including chemoprevention. In our study, benzo[a]pyrene-induced HT-29 colorectal cell line was used as the DME model. The activity of phase I enzyme CYP1A as determined by the 7-ethoxyresorufin O-deethylation (EROD) assay was found to be inhibited significantly by pterostilbene at 50  $\mu$ M, 75  $\mu$ M and 100  $\mu$ M ( $p \leq 0.01$ ,  $p \leq 0.05$ ,  $p \leq 0.01$  respectively) compared to the benzo[a]pyrene treated group. Meanwhile, pterostilbene induced glutathione-S-transferase (GST) activity significantly ( $p \leq 0.01$ ) at 50  $\mu$ M as compared to the untreated. In addition, However, the protein expression of CYP1A1 and GST in pterostilbene treated group was not significantly affected compared to untreated. On the other hand, pterostilbene at 25 and 75  $\mu$ M were able to increase the protein expression of transcription factor Nrf2 significantly ( $p \leq 0.01$ ). Results indicated that pterostilbene could reduce metabolic activation of procarcinogens and increase the detoxification process which can be potentially developed as chemopreventive agent.*

**Keywords:** Pterostilbene; Cytochrome P450 1A1; Glutathione S-Transferase; Benzo[a]pyrene; HT-29 colorectal cell line

### ABSTRAK

*Enzim Metabolisma Dadah (EMD) telah menjadi sasaran agen kemencegahan semulajadi untuk menghalang, merencat dan membalikkan proses karsinogenesis. Pterostilbene, analog kepada resveratrol telah dilaporkan mempunyai pelbagai manfaat farmakologi termasuk kemencegahan. Dalam kajian kami, sel HT-29 teraruh benzo[a]pirena telah digunakan sebagai model EMD. Aktiviti enzim CYP1A fasa I ditentukan oleh asai 7-ethoxyresorufin O-deethylation (EROD) didapati merencat secara signifikan oleh pterostilbene pada kepekatan 50  $\mu$ M, 75  $\mu$ M dan 100  $\mu$ M ( $p \leq 0.01$ ,  $p \leq 0.05$ ,  $p \leq 0.01$  masing-masing) berbanding dengan kumpulan yang dirawat dengan benzo[a]pirena. Selain itu, pterostilbene juga mengaruh aktiviti glutathione-S-transferase (GST) secara signifikan ( $p \leq 0.01$ ) pada kepekatan 50  $\mu$ M berbanding dengan kumpulan yang tidak dirawat. Di samping itu, pengekspresan protein CYP1A1 dan GST dalam kumpulan yang dirawat pterostilbene tidak memberi kesan secara signifikan berbanding kumpulan tidak dirawat. Sebaliknya, pterostilbene pada kepekatan 25  $\mu$ M dan 75  $\mu$ M dapat meningkatkan pengekspresan protein faktor transkripsi Nrf2 secara signifikan ( $p \leq 0.01$ ). Hasil kajian menunjukkan bahawa pterostilbene dapat mengurangkan pengaktifan metabolik prokarsinogen dan meningkatkan proses detoksifikasi yang berpotensi dapat dijadikan sebagai agen kemencegahan.*

**Kata kunci:** Pterostilbene; Sitokrom P450 1A1; Glutathione S-Transferase; Benzo [a] pirena; sel kanser kolorektal HT-29

### INTRODUCTION

In year 2010, approximately 7.98 million people died of cancer alone, a 28 % increment from the past 2 decades (Lozano et al. 2012). Colorectal cancer is the third highest cause of death among men and the second highest among women around the world (Ferlay et al. 2013). Epidemiology study had shown that environmental factors, such as diet play an important role in the susceptibility of colon cancer (Martinez 2005), with 80% of colorectal cancer cases contributed by the dietary factor (Bingham

2000). Polycyclic aromatic hydrocarbon, an environmental and dietary procarcinogen which can be found in barbequed meat has gained attention from researchers as it contributed toxicity to the gastrointestinal system. A study consisted of 1008 subjects had shown an increase in the risk of rectal adenoma with the intake of benzo[a]pyrene, a polycyclic aromatic hydrocarbon in the meat (Ferrucci et al. 2012).

In the wake of curbing the increasing trend of cancer cases, chemoprevention had been the focus of many researches for the past few decades. Chemoprevention was defined as the use of natural or synthetic or biological

agent to inhibit, retard and reverse the multistep molecular process of carcinogenesis (Sporn 1976). Phytochemicals could be a chemoprevention agent due to its nature to disrupt the process of carcinogenesis. According to Surh (2003), some phytochemicals could act as a cancer blocking agent or cancer suppressing agent. Phytochemicals, such as pterostilbene has also gained much attention in the field of cancer research due to its numerous health benefits (McCormack & McFadden 2012). Pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene) is a natural dimethylether analog of resveratrol that can be found in grapes and blueberries (Rimando et al. 2004). The presence of methoxyl functional groups in the phenolic ring increases its oral absorption and bioavailability which is greater than resveratrol. (Kapetanovic et al. 2011). Moreover, pterostilbene possess 7 times longer half-life than resveratrol (Remsburg et al. 2008). Due to its pharmacological benefits, pterostilbene is more favoured in the chemoprevention research compared to resveratrol. In vivo studies had also demonstrated the ability of pterostilbene in killing cancer cells, slowing the growth of cancer cells and inhibiting the initiation process of normal cells into precancerous cell under electrophilic stress (Schneider et al. 2010).

There are various mechanisms in chemoprevention that have been elucidated. In this present study, we mainly focused on the modulation of Drug Metabolizing Enzymes (DME), mainly phase I and II enzymes that involve in the detoxification of xenobiotics. Phase I DME, mainly the cytochrome P450 metabolic activates procarcinogens, such as benzo[a]pyrene to genotoxic electrophilic intermediates which can lead to cancer. Cytochrome P450 1A1 (CYP1A1) and its isoforms, CYP1A2 and CYP1B1 are able to metabolically activate procarcinogen benzo[a]pyrene into oxide and diol forms which can bind to DNA molecules and form DNA adduct, leading to the initiation of cancer (Ioannides & Lewis 2004). Phase II DME which consist of glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) help the process of conjugation of the electrophilic metabolites to a more water soluble derivative, which completes the cycle of detoxification (Guo et al. 2010). GST is the enzyme responsible for the cellular protection against the attack of reactive metabolites by conjugating the metabolites with glutathione (Sheweita & Tilmisany 2003). Regulation, expression and gene coding activities of phase I and II DME are the important factors in determining the propensity towards cancer (Nebert et al. 1996). The objective of chemoprevention can be achieved via the inhibition of metabolic activation of procarcinogen to ultimate carcinogen, at the same time, inducing the activity of phase II detoxification enzymes (Wattenberg 1996). Furthermore, cellular defences against chemical insults, whether of endogenous or exogenous origins, are regulated by transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which regulates the gene expression of phase II enzymes via the interaction

with Antioxidant Response Element (ARE) (Yu & Kensler 2005).

The objective of our study was to determine the effect of pterostilbene on the activities and proteins expression of phase I and II DME namely CYP1A1 and GST under the induction of benzo[a]pyrene. To give a better picture on the induction mechanism of phase II enzymes, the effect of pterostilbene on the protein expression of transcription factor Nrf2 was also investigated.

## MATERIALS AND METHODS

### MATERIALS

Human colorectal carcinoma cell line HT-29 was obtained from ATCC (Rockville, MD USA). Chemicals used for treatment were pterostilbene (C<sub>6</sub>H<sub>16</sub>O<sub>3</sub>), benzo[a]pyrene and resveratrol which were purchased from EMD Biosciences/Calbiochem® (USA). Chemicals for EROD assays composed of 7-ethoxyresorufin, resorufin, dicumarol, sodium acetate, β-glucuronidase/arylsulfatase and methanol together with chemicals for GST assay which were 1-chloro-2,4-dinitrobenzene (CDNB), reduced L-glutathione (GSH) and (5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (USA).

### CELL CULTURE

HT-29 cells were grown in modified McCoy's 5A media supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### CELL TREATMENT

The cells were incubated with pterostilbene at the concentrations of 25, 50, 75 and 100 μM and resveratrol at the concentration at 100 μM for 24 hours. After the 24 hours incubation, the cells were then induced with 10 μM benzo[a]pyrene (BaP) and incubated for 24 hours prior to harvesting. As for untreated control, the cells were cultured in the absence of pterostilbene for 24 hours followed by 10 μM BaP treatment for 24 hours prior to harvesting.

### 7-ETHOXYRESORUFIN O-DEETHYLATION (EROD) ASSAY

EROD assay was done to determine the activity of 7-ethoxyresorufin O-deethylation by CYP1A1 enzymes. The assay was done according to the method by Mingoia et al. (2007) with some modifications. The standard curve was first done with the 0-200 pmol resorufin. Briefly, the culture media with the treatments was removed and gently washed with PBS. Then, the cells were incubated with 100 μl PBS containing 8 μM 7-ethoxyresorufin and 10 μM dicumarol. The plates were then gently shaken to ensure even mixing followed by incubating in 37°C and 5% CO<sub>2</sub> incubator. After incubation, about 75 μl PBS in the wells

were aliquoted to black-coloured 96-well microtiter plate. About 10  $\mu$ l 0.5 M sodium acetate was pipetted into each well before adding 15  $\mu$ l  $\beta$ -glucuronidase/arylsulfatase which had been diluted with distilled water at 1:100 ratio (v/v). The plate was shaken gently before incubating for 2 hours at 37°C. The reaction of the enzyme was stopped by adding 100  $\mu$ l methanol in each well. Then, about 100  $\mu$ l of the mixture was then discarded and about 100  $\mu$ l 0.5 M glycine-sodium hydroxide was pipetted into each well. Finally, the concentration of resorufin was determined using spectrofluorometer at the excitation wavelength of 530 nm and emission wavelength of 590 nm. The EROD activity was calculated with the formula below and expressed in pmol/min/ $10^5$  cell.

#### GLUTATHIONE S-TRANSFERASES (GST) ASSAY

The GST activity in the samples was determined using GST assay (Habig and Jakoby 1981) which based on the rate of glutathione conjugation of 1-chloro-2,4,-dinitrobenzene (CDNB) as substrate to form product of S-(2,4-dinitrophenyl)-glutathione (GS-DNB). Then, 840  $\mu$ l phosphate buffer (100 mM  $K_2HPO_4$  and 100 mM  $KH_2PO_4$ ) pH 6.5 was used as blank. 100  $\mu$ l GSH (1 mM) and 50  $\mu$ l of the sample were pipetted into a 1 ml cuvette along with 840  $\mu$ l phosphate buffer. 10  $\mu$ l CDNB (75 mM) was then added into the reaction mixture to start mixing and the mixture was mixed immediately. The rate of reaction (abs/min) was read using UV spectrophotometer at 340 nm wave length for five minutes. The protein concentration of the samples was determined using Bradford assay (1976). The extinction coefficient of 9.6  $mM^{-1}cm^{-1}$  was used to calculate the GST activity of the samples.

#### WESTERN BLOT ANALYSIS OF CYP1A1, GST AND NRF2

Firstly, After determining the protein concentration in the samples were determined using Bradford assay. Then, the protein samples and protein standard were subjected to SDS-polyacrylamide gel electrophoresis (stacking gel, 5% w/v acrylamide; separation gel, 12% w/v acrylamide)

and transferred to PVDF membrane by using Bio-Rad Mini Transblot Module. The Western blots were subsequently blocked with blocking solution and incubated overnight with the primary antibodies such as CYP1A1 and GST which were diluted with 1: 1000 ratio with 5% skimmed milk. The blots were washed and incubated with secondary antibodies for 2 hours. Quantitative densitometric evaluation of protein bands was performed using a gel documentation Fusion FX which employed software FusionCapt Advance FX7, Fusion Ink. The blots were then washed and incubated with primary beta-actin mouse mAb, washed and then incubated with the secondary anti-mouse IgG, with both antibodies diluted in the ratio of 1:10,000 with 5% skimmed milk. The blots were then quantified using similar instrumentation as described as above.

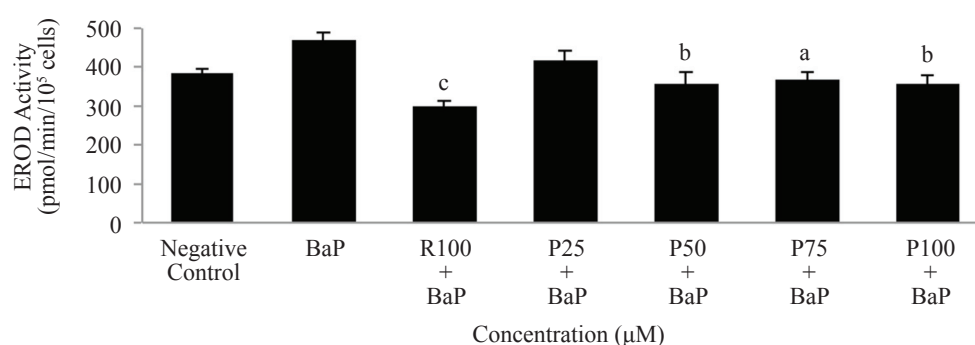
#### STATISTICAL ANALYSIS

Data were analysed using GraphPad Prism version 6.0 software (GraphPad Software, Inc. California, USA) and were presented as mean  $\pm$  S.E.M of the triplicate study. One-Way ANOVA was used to compare the treatment group with control. The comparison was deemed statistically significant when  $p \leq 0.05$ .

#### RESULTS AND DISCUSSION

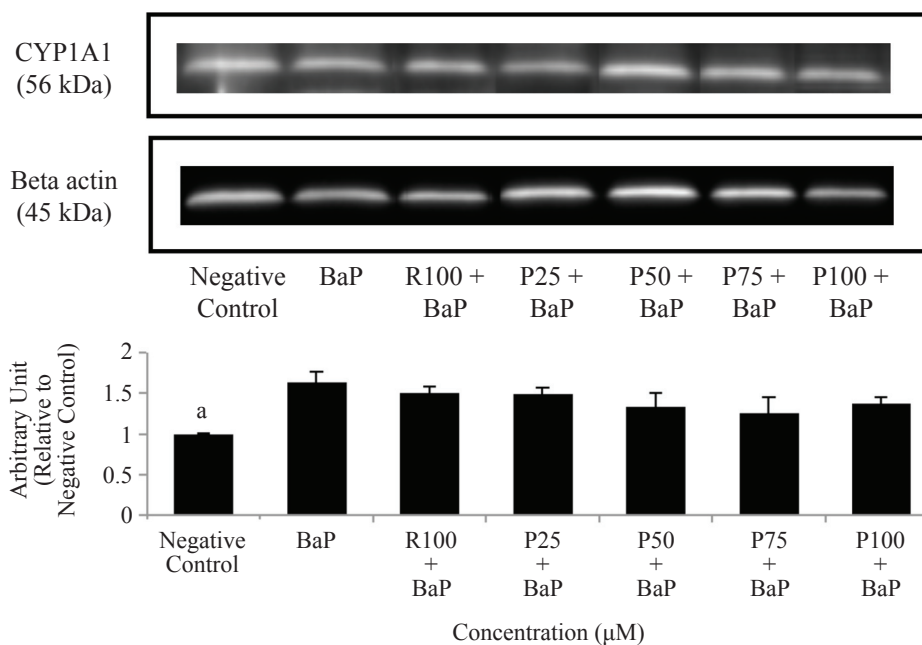
The effects of pterostilbene on the activity and protein expression of by CYP1A1 in benzo[a]pyrene-induced HT-29 cells.

As shown in both Figure 1 and Figure 2, benzo[a]pyrene at 10  $\mu$ M was able to increase the EROD activity and protein expression of CYP1A1 significantly compared to negative control. While significant inhibitory effect on EROD activity could be observed in the cells treated with pterostilbene at the concentration of 50, 75 and 100  $\mu$ M (Figure 1) comparably to the BaP control, pterostilbene was not able to inhibit the expression of CYP1A1 enzyme significantly at all concentration tested.



BaP: Benzo[a]pyrene; R: Resveratrol; P: Pterostilbene

FIGURE 1. EROD activity by CYP1A1 enzymes in benzo[a]pyrene-induced HT-29 cells of various experimental groups. Each value was represented by mean  $\pm$  S.E.M. (n = 3)



BaP: Benzo[a]pyrene; R: Resveratrol; P: Pterostilbene

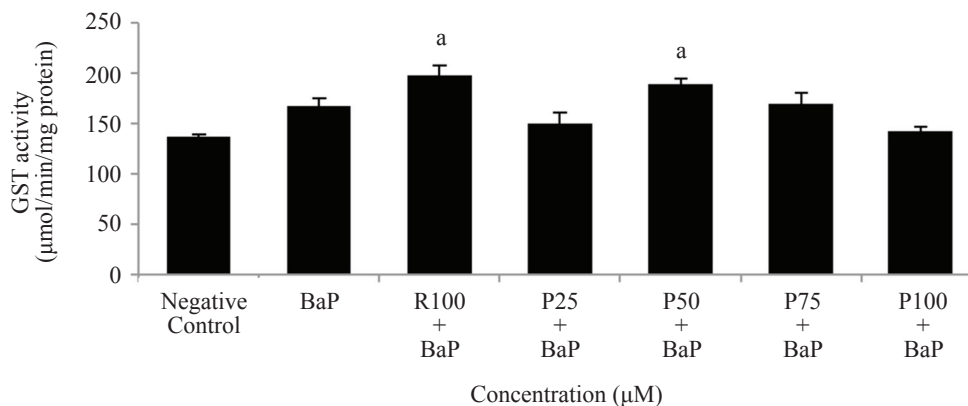
FIGURE 2. Protein expressions of CYP1A1 enzyme in benzo[a]pyrene-induced HT-29 cell of various experimental groups for 24 hours. Each value was represented by triplicate mean  $\pm$  S.E.M. (n = 3)

Benzo[a]pyrene can activate the gene transcription of CYP1A1 via the binding of benzo[a]pyrene with aryl hydrocarbon receptor nuclear translocator (ARNT) with the translocation of aryl hydrocarbon receptor (AhR) (Whitlock 1999). Our results were in agreement with Ciolino and Yeh, (1999) where they showed resveratrol had the ability to inhibit carcinogen activity by CYP1A1/1A2 enzymes in HepG2 cells competitively. As for pterostilbene, our results on pterostilbene treatment were in agreement with Ghazali et al., (2012) and Mikstacka et al., (2007). However, pterostilbene was not able to inhibit the protein expression of CYP1A1 significantly. Due to the short half-life of CYP1A1 mRNA, instability in translation and protein expression of CYP1A1 might occur (Lekas et al. 2000). Nevertheless, the inhibitory ability of pterostilbene on

EROD activity suggested that pterostilbene can be a potential chemopreventive agent with the inhibition of metabolic activation of procarcinogen, such as benzo[a]pyrene.

#### THE EFFECTS OF PTEROSTILBENE ON THE ACTIVITY AND PROTEIN EXPRESSION OF GST IN BENZO[A]PYRENE-INDUCED HT-29 CELLS

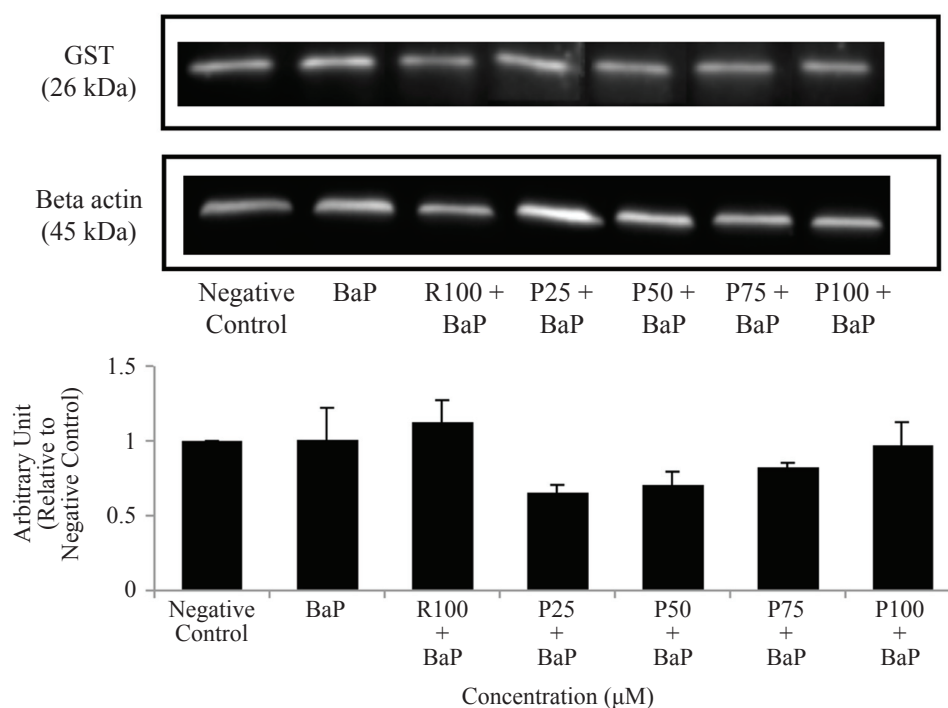
As shown in Figure 3, pterostilbene at all concentrations were able to increase the activity of GST, with significant effects observed in the cells treated with 50 μM pterostilbene compared to the negative control. The protein expression of GST was presented in Figure 4. Both resveratrol and pterostilbene did not show any significant effect on the protein expression of GST.



BaP: Benzo[a]pyrene; R: Resveratrol; P: Pterostilbene

FIGURE 3. GST activity in benzo[a]pyrene-induced HT-29 cell of various experimental groups. Each value was represented by triplicate mean  $\pm$  S.E.M. (n = 3)





BaP: Benzo[a]pyrene; R: Resveratrol; P: Pterostilbene

FIGURE 4. Protein expression of GST enzyme in benzo[a]pyrene-induced HT-29 cells of various experimental groups for 24 hours. Each value was represented by mean  $\pm$  S.E.M. (n = 3)

Our results were comparable to study by Ghazali et al. (2012) which showed an increase in GST activity after pterostilbene treatment. However, the increment was reduced with concentration of pterostilbene. This might be due to the cytotoxic effect exerted by pterostilbene at high concentration, as MTT assay demonstrated only 70 % cell viability was registered at 100  $\mu$ M pterostilbene. Even though pterostilbene was not able to increase the protein expression of GST significantly, its ability to induce GST activity proved that the potential of pterostilbene to increase glutathione conjugation of electrophilic xenobiotics for detoxification, reducing the risk of carcinogenic initiation process.

#### THE EFFECTS OF PTEROSTILBENE ON THE PROTEIN EXPRESSION OF NRF2 IN BENZO[A]PYRENE-INDUCED HT-29 CELLS

Figure 5 showed that pterostilbene at all concentrations were able to increase the protein expression of transcription factor Nrf2 in benzo[a]pyrene-induced HT29 cells compared to negative control, with a significant increment at 25  $\mu$ M and 75  $\mu$ M pterostilbene treated group.

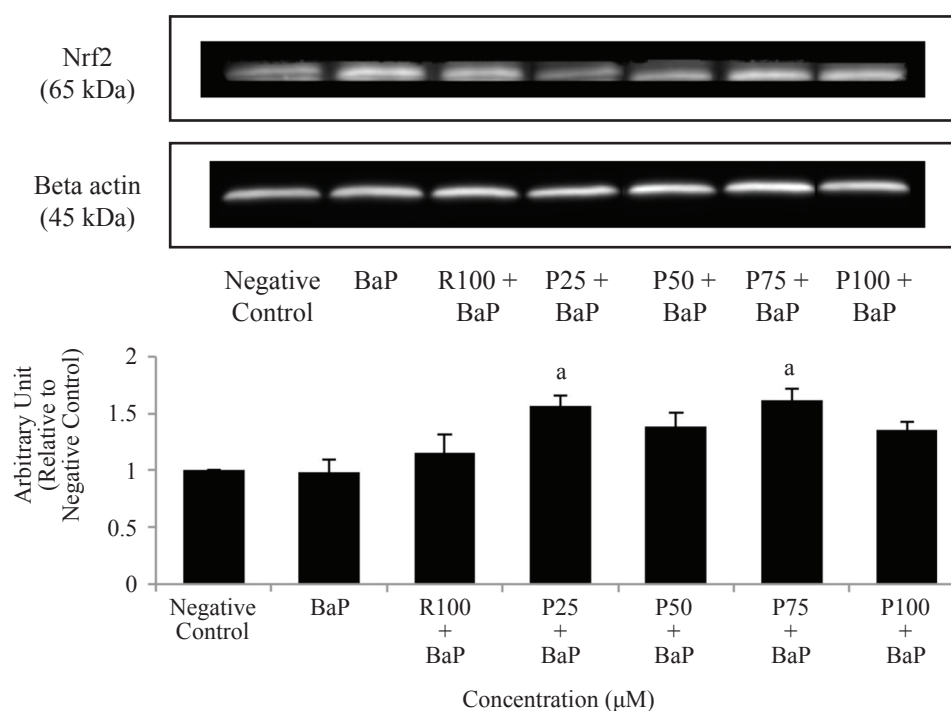
Nuclear factor erythroid 2-related factor 2 (Nrf2) plays an important role in preventing xenobiotic-induced toxicity and carcinogen-related tumorigenesis. The cellular protection effect is mainly due to the induction of phase II drug metabolizing enzymes or antioxidant enzymes via Nrf2-antioxidant response element (ARE) pathway (Shen and Kong, 2009). Our study depicted that pterostilbene was

better in inducing Nrf2 compared to resveratrol. Results on pterostilbene treatment group were supported by Chiou et al. (2011) where it showed that the induction of Nrf2 was greater by pterostilbene compared to resveratrol in a colon carcinogenesis rat model.

With the induction of transcription factor Nrf2, the expression of protein expression of phase II enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase (GCL) and UDP-glucuronosyltransferase (UGT) will be increased. Nrf2 also regulates the gene expression of antioxidant protein, anti-inflammation protein, calcium homeostasis protein and signalling molecules (Lee et al. 2003). Our study suggested that pterostilbene could protect cells from the oxidative stress, inflammation, and environmental pollutant-induced cancer via a complex detoxification system which would involve the transcription factor nrf2.

#### CONCLUSION

Our study showed the potential role of pterostilbene in inhibiting EROD activity by CYP1A1 and increase GST activity in benzo[a]pyrene-induced HT-29 cells. Besides, pterostilbene can increase the protein expression of Nrf2. In conclusion, pterostilbene is a potential chemoprevention agent through modulation of phase I and II drug metabolizing enzymes' activities and enhancement of the protein expression of Nrf2.



BaP: Benzo[a]pyrene; R: Resveratrol; P: Pterostilbene

FIGURE 5. Protein expression of transcription factor nrf2 in benzo[a]pyrene-induced HT-29 cells treated with pterostilbene for 24 hours. Each value was represented by triplicate mean  $\pm$  S.E.M. (n = 3)

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