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Effects of Pterostilbene on O-deethylation and Glutathione Conjugation of Drug Metabolizing Enzyme Activities

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Abstract: Background: Pterostilbene, a natural analog of resveratrol possesses diverse pharmacological properties, including anticancer, antioxidant, antiinflammatory and antiproliferation. The strategy of chemoprevention is generally through inhibition of phase 1 enzyme and induction of phase 2 enzyme activities. The research described in the present study was aimed at evaluating the effects of pterostilbene in modulation of drug metabolizing enzymes activities in chemoprevention. In this study, the direct effect of pterostilbene towards cytochrome P450 (CYP450) activity using microsomal fraction, Glutathione S-transferase (GST) activity and Glutathione (GSH) level using cytosol fraction of Chang liver cells were determined. Besides, cellular GST activity and GSH level were also determined after treatment of HepG2 cells with pterostilbene. **Results:** Results showed that pterostilbene was able to inhibit O-deethylation of 7-ethoxycoumarin activity significantly compared to control. In addition, pterostilbene also appeared to be more potent CYP450 inhibitor compared to resveratrol with percentage of 7-ethoxycoumarin O-deethylation (ECOD) activity of 49.58 and 74.79%, respectively. Results also indicated that chemical modulation of the stilbene backbone of resveratrol lead to the stronger inhibitory effect in pterostilbene. Whereas, for GST and GSH assays, it was found that pterostilbene did not posses direct effects on both GST activity and GSH level. However, pretreatment of HepG2 cells with pterostilbene had led to significant induction of GST activity as well as significant increment of GSH level. **Conclusion:** In conclusion, pterostilbene was able to act as a chemopreventive agent through modulation of drug metabolizing enzyme activities.

Key words: Pterostilbene, chemoprevention, O-deethylation, cytochrome P450, glutathione S-transferase

INTRODUCTION

According to World Health Organization, cancer is a leading cause of death worldwide (WHO, 2008). According to statistics, the cancer ratio for Malaysians is one to four and if no action taken, one out of three will acquire cancer (BCWA, 2009). Carcinogenesis can be regarded as the accumulation of chemical or biological substances that causes damage to normal cells, thus changing the genetic levels that lead to promotion, propagation and eventually, cancer (Hong and Sporn, 1997). This multistage disease offers a variety of targets for chemopreventive agents to prevent tumor initiation or inhibit tumor development.

Chemoprevention is defined as the use of natural or synthetic substance to halt, retard or reverse the promotion stage of carcinogenesis (Bagchi and Preuss, 2005). It is believed that diets of fruits and vegetable rich in phytochemicals were able to interrupt carcinogenesis (Surh, 2003). Phytochemicals, particularly pterostilbene, are of great interest as chemopreventive agent due to its potential health benefits (Wilson *et al*., 2008). Pterostilbene (trans-3, 5-dimethoxy-4'-hydroxystilbene), an analogue of resveratrol found in grapes and blueberries (Rimando *et al*., 2004), appeared potentially useful in chemoprevention (Rimando *et al*., 2002). Besides, pterostilbene also possessed various pharmacological properties, such as antioxidant, anticancer, antidiabetic and antifungal (Roupe *et al*., 2004). Pterostilbene had showed significant increase in antioxidant activity due to the presence of hydroxyl group (Hasiah *et al*., 2011) and increase in bioavailability due to the presence of methoxy group (Mikstacka *et al*., 2007).

There are several mechanisms in chemoprevention. An important research method to unfold the potentials of a chemopreventive agent is through modulation of drug

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metabolizing enzymes that were involved in xenobiotic metabolism which includes phase 1 and 2 drug metabolizing enzyme activities (Talalay *et al*., 1995). Phase 1 reactions involve changes in molecular structure through mechanisms such as oxidation and dealkylation while phase 2 reaction involves conjugation activities which increase the polarity and solubility of one compound (Kerns and Li, 2008). 7-Ethoxycoumarin O-deethylation (ECOD) assay was used to determine overall CYP450 activity in this study. ECOD assay is a sensitive spectrofluorimetric method which uses 7-ethoxycoumarin as substrate (Waxman and Chang, 2006). However, CYP450 activity increases the risk of producing ultimate carcinogens in which these reactive species were capable of reacting with DNA and might initiate carcinogenesis. Thus, induction of phase 1 or bioactivation of xenobiotics requires complete detoxification by phase 2 reactions; in this case Glutathione S-transferase (GST) and Glutathione (GSH) (Gerhauser *et al*., 2003). GST is an essential enzyme in protecting the cells from reactive species and oxidative stress by conjugating the reactive species with GSH (Kwon, 2001). Generally, the strategy to prevent tumor initiation is through inhibition of phase 1 and induction of phase 2 enzymes (Gerhauser *et al*., 2003).

The aim of the study was to determine the effect of pterostilbene on drug metabolizing enzymes activities that is inhibitory effect of pterostilbene towards CYP450 and inductive effect of pterostilbene towards GST and GSH.

MATERIALS AND METHODS

Materials: Human Chang liver cells and HepG2 cells were obtained from ATCC (Rockville, MD USA). RPMI 1640 and DMEM were purchased from invitrogen Corporation (USA) and Foetal Bovine Serum (FBS), Penicillin-Streptomycin and trypsin-EDTA were purchased from PAA (Austria). Compounds used in this research were pterostilbene and resveratrol which were purchased commercially from EMD Biosciences (Germany). 7-Ethoxycoumarin was from Acros Organics (USA) while β-nicotinamide-adenine dinucleotide (β-NADH), β-nicotinamide adenine dinucleotide phosphate, reduced tetra (cyclohexylammonium) salt (β-NADPH), 7-hydroxycoumarin, 1-chloro-2,4-dinitrobenzene (CDNB), reduced Glutathione (GSH) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (USA).

Cell culture: Human Chang liver cells and HepG2 cells were grown as monolayer in T-25 cm^2 culture flask. Chang liver cells (average doubling time is 16.8 h) and HepG2 cells were maintained in RPMI 1640 medium and DMEM, respectively. RPMI 1640 medium was added with 2.0 g L^{-1} sodium bicarbonate while DMEM was added with 1.5 g L^{-1} sodium bicarbonate. Both media were then supplemented 1% Penicillin/Streptomycin and 10% Fetal Bovine Serum (FBS). The cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C and harvested when the cell confluence reached 80%.

Subcellular fractionation of Chang liver cells: Chang liver cells with concentration of 5×10^4 cells mL^{-1} were cultured in 15 mL medium in culture dish. The cells were scrapped out when they reached 80% confluency and collected into centrifuge tube and then pelleted at 220×g for 5 min. The supernatant was discarded and the pellets was washed with chilled PBS before centrifuged again at 220×g at 4°C for 5 min. The pellet was mixed with 100 μL of ice cold lysis buffer (50 mM K_2HPO_4, 1 mM EDTA, pH 6.5 and 0.1% v/v Triton X-100) and incubated on ice for 15 min before centrifuged at 10,000×g at 4°C for 15 min. Subsequently, the supernatant was transferred into Optiseal™ ultracentrifuge tube for ultracentrifugation at 100,000xg at 4°C for 1 h. The cytosol fractions were transferred out and the microsomal fractions (pellet) were added with Tris buffer (0.1 M Trizma HCl, 0.1 M Trizma base and 0.15 M KCl, pH 7.4). All fractions were stored at -80°C before used.

Determination of 7-Ethoxycoumarin O-deethylation (ECOD) activity: CYP450 activity was determined using 7-ethoxycoumarin O-deethylation (ECOD) described by Greenlee and Poland (1978) with slight modification. Five sets of tubes (0-100 μM pterostilbene and resveratrol 100 μM) consisted of blank, test and standard tubes were prepared. The reaction mixtures contain 65 μmol potassium phosphate buffer, 0.50 μmol β-NADPH, 0.50 μmol β-NADH, 5 μmol $MgCl_2$, 1 mg BSA, 0.03 mL microsomal fraction, 20 μL pterostilbene (0-100 μM) and distilled water with total volume of 1.0 mL. The mixture was then incubated in shaking water bath at 100 rpm., 37°C for 5 min. Then, the reaction was started by adding 200 μL 7-ethoxycoumarin 10 mM (in 50% aqueous methanol) as substrate and the mixture was incubated in shaking water bath for 10 min. The reaction was terminated by addition of 0.125 mL of 15% (w/v) trichloroacetic acid and 2 mL of chloroform in test and standard tubes while 0.5 mL of 4 M hydrochloric acid and 6 mL chloroform were added in the blank tube. Fifty microliter of 7-hydroxycoumarin were added to the standard tube to produce 0.0025 mM 7-hydroxycoumarin in each standard tubes. All the tubes were then

incubated in shaking water bath C for 10 min so, that 7-hydroxycoumarin was partitioned into the chloroform layer. Then, 1.0 mL of organic phase was transferred into 5 mL test tube, followed by addition of 3.0 mL 0.01 N NaOH-1 M NaCl solution. Alkaline phase were then transferred into quartz cuvette to determine the concentration of 7-hydroxycoumarin using Shimadzu RF-1501 spectrofluorometer (GmBH, Europe) at excitation wavelength of 368 nm and emission wavelength of 456 nm. The ECOD activity was calculated using the formula below and expressed in nmole/min/mg protein. Protein concentration was determined by Bradford assay (Bradford 1976).

$$\frac{\text{Fluorescence (test - blank)} \times \text{2.5 nmol 7-hydroxycoumarin in standard tube}}{\text{10 min} \times \text{0.03 mL} \times \text{protein concentrationxfluorescence (standard-blank)}}$$

Determination of glutathione S-transferase (GST) activity: GST activity was measured using the method described by Habig and Jakoby (1981). Each cuvette contained 830 μL of phosphate buffer pH 6.5 (100 and 100 mM KH_2PO_4), 10 μL pterostilbene (0-100 μM), 100 μL of 1 mM glutathione and 50 μL of cytosol fraction. About 10 μL CDNB was added to start the reaction. The change of absorbance was measured for 3 min at 340 nm using Uvi Light spectrophotometer (Secomam, France). GST activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1} and expressed as μmole/min/mg protein. Protein concentration was measured using the Bradford assay (Bradford, 1976).

Determination of glutathione (GSH) level: GSH level was quantified as described by Ellman (1959). Cytosol fraction (50 μL) and GSH standards which consist of serial dilution of 10 mM GSH dissolved in reaction buffer (0.1 M $Na_2HPO_4 \cdot 7H_2O$ and 1 mM EDTA, pH 6.5) were added into wells in 96-well plate. GSH standards wells were added with 40 μL of reaction buffer (0.1 M $Na_2HPO_4 \cdot 7H_2O$ and 1 mM EDTA, pH 8) while 30 μL of reaction buffer and 10 μL pterostilbene (0-100 μM) were added into samples wells. Then 10 μL of 4 mg mL^{-1} DTNB (in reaction buffer, pH 8) were added to all wells and absorbance was measured at 405 nm using ELISA microplate reader (Biochrom ASYS Expert Plus, UK) after incubated for 15 min at 37°C. The concentration of GSH in the samples was determined from GSH standard curve and expressed as μmole/mg protein. Protein concentration was measured using the Bradford assay (Bradford, 1976).

Determination of cellular glutathione S-transferase (GST) activity and glutathione (GSH) level in HepG2 cells: HepG2 cells with concentration of 5×104 mL^{-1} were cultured in 15 mL medium in culture dish for 24 h. The cells were then incubated in the presence of pterostilbene (0-100 μM) for 24 h. After this time of periods, the cells were harvested and lysed as methods described above. Then, the cytosol fractions were used in GST and GSH assays to determine GST activity and GSH level respectively.

Statistical analysis: All data were expressed as Mean±SEM (standard error of mean). Statistical analysis was then performed using SPSS Version 18.0. Analysis of variance (ANOVA) was used to compare the effect of pterostilbene between control and treatment. Statistical significance was considered at $p<0.05$.

RESULTS

Effects of pterostilbene on 7-ethoxyoumarin O-deethylation (ECOD) activity: Effects of pterostilbene on cytochrome P450 activity was determined using 7-ethoxyoumarin O-deethylation assay, which is useful in determining overall CYP450 activity. To test the direct inhibitory effect of pterostilbene on ECOD activity, microsomal fraction isolated from Chang liver cells was used. As shown in Fig. 1 microsomal fractions treated with 25-100 μM pterostilbene had resulted significant decrease in CYP450 activity in a concentration dependent manner. Pterostilbene showed highest inhibition on ECOD activity at 100 μM. Besides, these results were also compared to resveratrol (100 μM) which had showed inhibition in ECOD activity. Effects of 50, 75 and 100 μM pterostilbene had shown significant decrease in ECOD activity compared to 100 μM resveratrol.

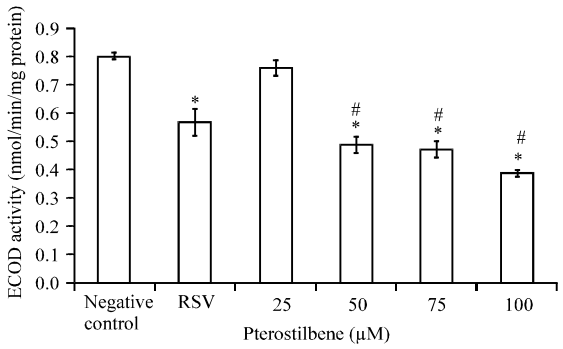

Fig. 1: Effect of pterostilbene on CYP450 activity of microsomal fraction of Chang liver cells was determined. n = 3 ±SEM, *Significant compared to negative control and (#) compared to 100 μM resveratrol ($p<0.05$)

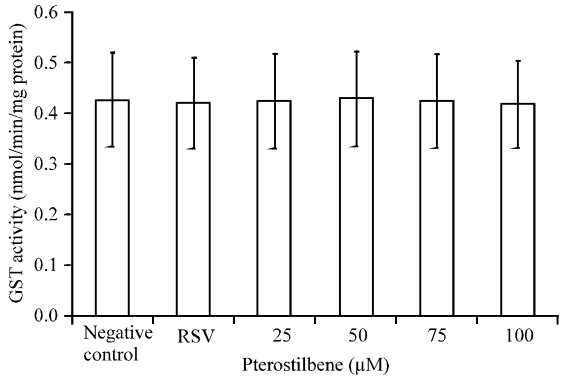

Fig. 2: Effect of pterostilbene on GST activity of cytosol fraction of Chang liver cells n = 3 ±SEM

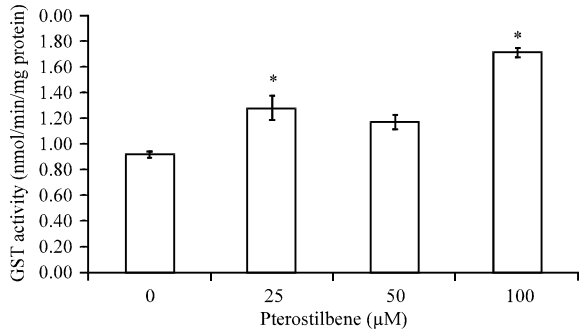

Fig. 3: Effect of pterostilbene on GST activity in HepG2 cells n = 3 ±SEM, *Significant compared to negative control

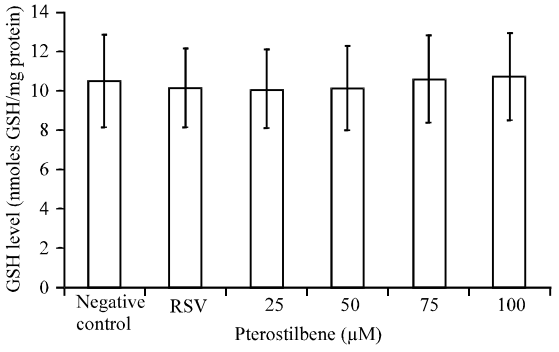

Fig. 4: Effect of pterostilbene on GSH level in cytosol fraction of Chang liver cells n = 3 ±SEM

Effects of pterostilbene on GST activity: In order to test the direct inductive effect of pterostilbene on GST activity, cytosol fraction isolated from Chang liver cells was used. Pterostilbene showed no direct effect on GST activity as shown in Fig. 2. However, incubation of HepG2 cells with pterostilbene had led to mark increase in cellular GST activity with highest inductive effect at 100 µM (Fig. 3).

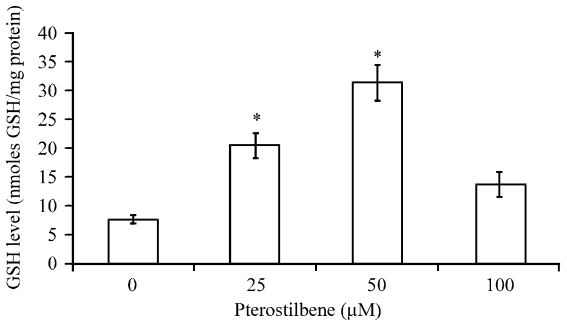

Fig. 5: Effect of pterostilbene on GSH level in HepG2 cells n = 3 ±SEM, *Significant compared to negative control

Effects of pterostilbene on GSH level: In this study, effect of pterostilbene on GSH level was also examined. There was no direct effect on GSH level when cytosol fraction of Chang liver cells was treated with different concentrations of pterostilbene, as shown in Fig. 4. However, there was a significant increase in GSH level when HepG2 cells were treated with pterostilbene during seeding (Fig. 5).

DISCUSSION

Chemopreventive phytochemicals are non toxic agents that are able to halt or reverse cancer development and retard the progression of carcinogenesis to form malignant tumour (Surh, 2003). Pterostilbene is one of the naturally occurring phytochemicals with various pharmacological functions (Rimando and Suh, 2008). Modulation of drug metabolizing enzymes, which is through inhibition of phase 1 enzymes concurrent with activation of phase 2 enzymes are important strategies in preventing cancer (Gerhauser *et al*., 2003).

CYP450 is one important drug metabolizing enzymes in the liver (Rodriguez-Antona *et al*., 2002) and are able to increase the risk of producing ultimate carcinogens which cause DNA damage and eventually cancer (Gerhauser *et al*., 2003). 7-Ethoxycoumarin O-deethylation (ECOD) assay was because of its sensitivity and ability to determine the overall activities of CYP450 (Castell *et al*., 2005). 7-Ethoxycoumarin is a common substrate that can be metabolized by several enzymes of CYP450 family, thus useful in determining enzyme activities of CYP1, CYP2 and CYP3 (Waxman and Chang, 2006). From the results obtained, pterostilbene was able to inhibit CYP450 activity significantly when microsomal fraction of Chang liver cells was treated with pterostilbene. Besides, pterostilbene also showed more significant inhibition in CYP450 activity compared to resveratrol. Thus, it was obvious that there was increase in inhibitory effect on CYP450 activity when hydroxyl group of resveratrol was

replaced by methoxy group. This replacement also increased the lipophilic properties of resveratrol analog, thereby increasing the bioavailability of that compound (Mikstacka *et al*., 2007). Several studies also indicated that biological activities including antioxidant activities and bioavailability of stilbene depends on their structure (Hasiah *et al*., 2011). Chemical modification of stilbene backbone was significantly affecting the biological activities of stilbene; in this case, ECOD activity by CYP450. Therefore, pterostilbene was more potent CYP450 inhibitor than resveratrol.

GST is one of the major detoxification enzymes in phase 2 reactions which plays an important role in detoxifying and protects the body from oxidative stress (Jancova *et al*., 2010) by catalyzing the conjugation of reactive species to GSH (Kwon, 2001). In this study, GST activity, pterostilbene had shown no direct effect on GST activities. However, incubation of HepG2 cells with pterostilbene had led to the increase in GST activities. Through the treatment of pterostilbene onto the cells, GST enzyme will be produced as a result of the activation of Nrf2 (Nuclear factor erythroid 2-related factor 2), which is a transcription factor that is important in regulating expression of various detoxification enzymes including GST. This is controlled by antioxidant response element (ARE) where Nrf2 will bind to ARE when translocated into nucleus, thus activating the transcription of the genes (Ramos-Gomez *et al*., 2001). Pterostilbene might also increase the Nrf2 levels in both nucleus and cytoplasm as shown by resveratrol in studies done by Rubiolo *et al*. (2008).

Lastly, GSH is important as an antioxidant by reacting with reactive electrophiles (Schulz, 2007). GSH assay conducted on cytosol fractions of Chang liver cells showed no increase in GSH levels after addition of pterostilbene. However, by treating HepG2 cells with pterostilbene, there was significant increase in GSH levels but not in concentration dependent manner. This is through the increase expression of Glutamate-cysteine-Ligase (GCL) which is activated by Nrf2 and thus increasing the synthesis of GSH (Kode *et al*., 2008). This results was compatible to other studies by using resveratrol. Resveratrol had led to the increase in GSH level after incubation of cells with three different periods (24, 48 dan 72 h) (Cao and Li, 2004) and was also able to restore GSH level after cigarette smoke-mediated GSH depletion through activation of Nrf2 (Kode *et al*., 2008).

Overall, pterostilbene was able to reduce CYP450 activity but did not exert direct effects towards GST activity and GSH levels. However, pretreatment of HepG2 with pterostilbene had led to the increase in both cellular GST activity and GSH levels. In short, pterostilbene was able to modulate enzyme activities as one of the strategies in chemoprevention. These approaches protect the body from carcinogens through inhibition of phase 1 reactions, whereby reducing the risk of activating procarcinogens into carcinogens (Kelloff *et al*., 2005) and induction of phase 2 reactions, whereby increasing detoxification and secretion of reactive species (Lewis *et al*., 2010). Thus, phase 2 induction is vital as indirect antioxidant in neutralization of reactive species and exerts protection against various carcinogens (Ramos-Gomez *et al*., 2001).

This study showed that pterostilbene was capable in inhibiting ECOD activity and was a better CYP450 inhibitor than resveratrol. Besides, pretreatment of HepG2 cells with pterostilbene also led to an increase in both cellular GST activity and GSH level. Overall, the inhibition of phase 1 reactions and induction of phase 2 reactions showed that pterostilbene is able to act as a chemopreventive agent through modulation of drug metabolizing enzymes activities.

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