Histopathological effect of pterostilbene as chemoprevention in N-NITROSO-TRI-CHLOROETHYLUREA (NTCU)-Induced lung squamous cell carcinoma (SCC) mouse model

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**Title:** HISTOPATHOLOGICAL EFFECT OF PTEROSTILBENE AS CHEMOPREVENTION IN N-NITROSO-TRI-CHLOROETHYLUREA (NTCU)-INDUCED LUNG SQUAMOUS CELL CARCINOMA (SCC) MOUSE MODEL.

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ABSTRACT

**Background:** Lung cancer is the leading cause of cancer-related deaths, and squamous cell carcinoma (SCC) is one of the most common types of lung cancer. Chemoprevention of lung cancer has gained increasing popularity as an alternative to treatment in reducing the burden of lung cancer. Pterostilbene (PS) may be developed as a chemopreventive agent due to its pharmacological activities, such as anti-proliferative, anti-inflammatory and antioxidant properties. This study aimed to investigate the effect of PS on the development of lung SCC in the mouse model. **Methods:** A total of 24 seven-week-old female Balb/C mice were randomly categorised into four groups, including two control groups comprising the N-nitroso-trischloroethylurea (NTCU)-induced lung SCC and vehicle control (VC) groups and two treatment groups comprising the 10mg/kg PS (PS10) and 50mg/kg PS (PS50) groups. All lung organs were harvested at week 26 for histopathological analysis. **Results:** All PS treatment groups showed chemopreventive activity by inhibiting the progression of lung SCC formation with PS10, resulting in mild hyperplasia, and PS50 was completely reversed in the normal bronchial epithelium layer compared with the VC group. PS treatment also reduced the expression of cytokeratin 5/6 in the bronchial epithelium layer. Both PS10 and PS50 significantly reduced the epithelium thickness compared to the NTCU group (p<0.05). PS is a potential chemopreventive agent against lung SCC growth by suppressing the progression of pre-malignant lesions and reducing the thickness of the bronchial epithelium. **Conclusions:** The underlying molecular mechanisms of PS in lung SCC should be further studied.

**Keywords:** pterostilbene, lung cancer, squamous cell carcinoma (SCC), chemoprevention, histopathology, cytokeratin 5/6
INTRODUCTION

Lung cancer has a very high mortality rate and is the leading cause of cancer-related death (WHO, 2018a). Based on histological observations under the light microscope, lung cancer can be divided into two major groups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLCs can be divided into a few subtypes, such as adenocarcinoma (AD), lung squamous cell carcinoma (SCC) and large cell carcinoma (LCC) (Campbell et al., 2016; Surien et al., 2019). Among all types of human lung cancers, the prevalence of lung AD and SCC is the highest (Siang and John, 2016; Pan et al., 2019). High mortality in patients with lung cancer is due to a late diagnosis in which most cases of lung cancer (70%) have been diagnosed at advanced stages with tumours that cannot be surgically removed (Travis, 2011). Late diagnosis of tumours presented in advanced stages increases the risk of failure of treatment, such as chemotherapy and radiotherapy (Breuer et al., 2005). Lung cancers can be caused by various aetiologies, such as inborn genetic defects, the environment and lifestyle. Among these aetiologies, the environment and lifestyle are more closely associated with lung cancer than with genetic factors, which play a minor role in the development of lung cancer (Weidepass, 2010; Heikkila et al., 2013). Some lifestyles that may increase the risk of lung cancer are diet, obesity, and smoking (Cruz et al., 2011). Cigarette smoking is a well-established cause of all types of human lung cancer, but it has a very strong association with the occurrence of lung SCC and SCLC (Khunder, 2011).

Screening for the early detection of lung cancer is one of the key intervention steps to reduce mortality in which the majority of patients with early-stage lung cancer have shown high survival rates. However, the implementation of lung cancer screening has faced many challenges, such as the complexity of screening procedures (follow-up requirements) and the massive effort required by health workers and patients (Blandin et al., 2017; Kinsinger et al., 2017). Moreover, exposure to radiation and the cost of screening tests are patients’ concerns that may cause them to refuse screening (Mishra et al., 2014). Apart from early detection, chemoprevention has shown increasing popularity as an alternative approach to combat cancer. However, no chemopreventive agent has been shown to be effective in the prevention of lung cancer during clinical trials. In addition, improvement in the survival rate of patients with breast and prostate cancer has been reported in the last few decades due to advances in cancer management and treatment, while the survival rate of patients with lung cancer remains unchanged (Kim et al., 2012; DeSantis et al.,
An effective chemopreventive agent that can work synergistically with early detection should therefore be identified to reduce the morbidity and mortality of lung cancer (New and Keith, 2018). Cancer is a preventable disease. Therefore, intervention to reduce the risk of lung cancer is an alternative to overcoming the burden of lung cancer. Modifying lifestyles and dietary habits could reduce the risk of developing cancer. For example, the consumption of fresh fruits and vegetables among smokers may reduce the risk of developing lung cancer and thus prevent the development of lung SCC (Bucher et al. 2010). Lung SCC is the abnormal growth of bronchial epithelial cells with the most common histological features, such as the formation of keratin pearl and intercellular bridge (Perez-Moreno et al., 2012). Fresh fruits and vegetables are rich in polyphenols, which have chemopreventive effects, due to their ability to enhance the detoxifying enzymes and antioxidant properties (Amararatna et al., 2016). Chemoprevention is defined as the process of inhibition, suppression, or reversion of carcinogenesis or the development of cancer by using any natural or synthetic agent (Sporn et al., 1976).

Pterostilbene (PS) is a natural compound that belongs to the polyphenol stilbene group and can be found in blueberries, grapes and *Pterocarpus marsupium* trees (Roupe et al., 2006). Recently, PS has gained increasing scientific attention due to its various pharmacological activities related to health benefits (Reinisalo et al., 2015). PS plays a number of pharmacological roles, such as anti-inflammatory, antioxidant, anti-cancer and chemoprevention activities (Paul et al., 2009; Ghazali et al., 2012). PS has a similar chemical structure to its natural analogue compound, namely resveratrol, but PS has some advantages, with a higher bioavailability and a longer half-life (Remsberg et al., 2008; Lin et al., 2009). The toxic effects of PS were tested in animal studies in which the high dose (3,000 mg/kg) in the Swiss mouse did not cause any signs of toxicity, with normal biochemical parameters and histopathological tests on the systemic organs (Ruiz et al., 2009). Moreover, a dose of up to 250 per day of PS can be used safely in humans without causing any adverse effects (toxicity) (Riche et al., 2013). This study was therefore designed to investigate the chemoprevention effect of PS on lung SCC by using an in vivo model by observing the histopathological evaluation.
MATERIALS AND METHODS

Chemicals

The N-nitroso-tri-chloroethylurea, NTCU was purchased from Toronto Research Chemicals, Inc. The pterostilbene compound with purity 98% was obtained from Friedemann Schmidt Chemicals.

Animal Model of Lung SCC

A total of 24 Balb/c mice at 7 weeks of age were randomly divided into four groups (n=6 animals per group). All the mice were obtained from the Faculty of Veterinary of University Putra Malaysia (UPM), and only after two weeks after arrival were the mice used for research to ensure that the animals had adapted to new living conditions. The first group was the vehicle control VC group, which was treated with intraperitoneal (i.p) corn oil, followed by topical application of 70% acetone after 30 minutes in the shaved dorsal subscapular area. Pterostilbene was dissolved in corn oil, and NTCU was dissolved in 70% of acetone as a vehicle. The second group of mice was the NTCU group, which was treated with corn oil and 25 µL of 0.04M NTCU. The third and fourth groups were the pterostilbene treatment group, with a low dose of 10 mg/kg pterostilbene diluted in corn oil (PS 10) and a high dose of 50 mg/kg of pterostilbene diluted in corn oil (PS 50) respectively. The two treatment groups were also treated with 25 µL of 0.04M NTCU. Treatment took place twice a week with an interval of 3.5 days for 26 weeks. For the pterostilbene treatment groups, the first two weeks were the pre-treatment with pterostilbene without NTCU. After two weeks of pre-treatment with pterostilbene, we started to treat the mice with NTCU together with pterostilbene treatment until week 26, as shown in Figure 1. After 26 weeks of treatment, all the mice were sacrificed by cervical dislocation following an overdose of KTX (ketamine and xylazine) cocktails.

Haematoxylin & Eosin (H & E) staining

After the mice were sacrificed, the lung tissues were collected and stored in 10% buffered formalin for not more than 24 hours at 4°C. After fixation with formalin, the lungs tissue was kept in 70%
ethanol at 4°C until the next phase of autonomic dehydration and filtration by the autonomic tissue processor. After going through the autonomic tissue processor, the lung tissue was embedded in paraffin and sectioned into 4µm thick sections. After sectioning, the lung tissue was mounted on a microscope slide and kept in the oven for 24 hours to dry before proceeding with the H&E staining procedure. Image J software (Image J version 1.46r; National Institute of Health, Bethesda, United States of America, USA) was used to measure the thickness of the bronchial epithelium layer from the lung H&E staining of all mice for each group (n=6).

**Histopathological scoring and percentage of mice based on lung SCC carcinogenesis stages**

The tumour count was replaced with scoring on the type of histopathological lesions based on the lung SCC carcinogenesis. Macroscopic observation on the surface of the lung to detect solid nodular tumours is difficult to see and count because the lung SCC mouse model does not form a visible nodular tumour on the surface of the lung. Carcinogenesis of lung SCC is the multistage process that begins with the transformation of the normal bronchial epithelium layer into hyperplasia, metaplasia, dysplasia, and finally into carcinoma or lung SCC (Wang et al., 2009). By observing the H&E staining of the lungs, the type of lesions on the bronchial epithelium layer for each mouse was determined on the basis of the lung carcinogenesis stages mentioned above and proceeded with histopathological scoring and the percentage numbers of mice. H&E staining of the lungs of all mice was performed for each group (n=6). For each mouse, there are three areas (slides) under 40x magnification that focus on the bronchial epithelium layer that have been scored to obtain the mean histopathological score for that mouse. Histopathological scores based on lung SCC multistep carcinogenesis details are shown in Table 1.

**Immunohistochemistry staining**

Immunohistochemistry staining was used to study the expression level of cytokeratin 5/6 (Biorbyt, United States, Catalogue no: orb10411) in the bronchial epithelium layer. Paraffin-embedded lung tissues were sectioned with 4 µm thickness and placed on the adhesive charged glass slides. The lung tissues on the glass slides have been stained with cytokeratin 5/6 as a primary antibody with
a 1:300 dilution factor. Horseradish peroxidase (HRP) was used as a secondary antibody for the detection of complex antigen-antibody in lung tissues with brown colour staining with a 1:100 dilution factor. The FIJI/Image J software (Java 8 version 64-bit) was used for the quantification analysis of the expression of cytokeratin 5/6 in the lungs (Crowe and Yue, 2019). Measurement of percentage or proportion of stained areas (pixel intensity) on lung tissue by FIJI/Image J was performed on the immunohistochemistry staining images with 40x magnification.

**Statistical Analysis**

All values are stated as the mean ± standard error mean (SEM). The data for each group was normally distributed with a Shapiro-Wilk test using SPSS software version 22.0. One-Way ANOVA with Tukey’s post-hoc test was used to compare the variable between groups and the value of p< 0.05 was considered statistically significant.

**RESULTS**

**Histopathological observation**

Figure 2(A) shows the normal bronchial airway tract with a single layer of epithelial cells of the representative mouse from the vehicle control (VC) group under low magnification (10x). Under high magnification (40x), Figure 2(B) shows the H&E staining of the lung from the representative mouse of the VC group with the normal histology of the bronchial epithelium layer having a single layer of uniform ciliated columnar epithelium cells and basally located nuclei. Moreover, the epithelial basement membrane remains intact. Figure 2(C) (arrows) shows a severe thickening of the bronchial epithelium layer and an invasion of the bronchial basement membrane that appears to be no longer intact in the NTCU group. At a high magnification of 40x in Figure 2(D), the single layer of the bronchial epithelium was replaced by stratified (multi-layered) epithelial cells. In addition, under 40x magnification, the histological characteristics of the lung SCC are shown, where the cells are large, flattened, irregularly shaped and disorganised. Also, the cells showed variable sizes of the nucleus, and most cells exhibited increased nucleus/cytoplasm (N:C) ratio with hyperchromasia (darkly stained nuclei), as shown by the area of the arrows in Figure 2(D).
For the treatment groups, the mice that received a low dose of PS 10 (10mg/kg) with NTCU caused only a mild thickening of the bronchial epithelium layer in Figure 2(E) (arrows) with the transformation of single to a bilayer of epithelial cells. At high magnification (40x) in the observation shown in Figure 2(F) the lung sample obtained from the mouse treated with a low dose of PS (10 mg/kg) with NTCU in the PS 10 group, only mild hyperplasia (HP) was observed with an increased number of normal cells without any changes in the shape of the nucleus, and the normal size and uniform size of the nucleus were maintained. The high-dose of PS with NTCU in the PS 50 group (50mg/kg) maintained a single layer of bronchial epithelial cells as shown in Figure 2(G). Under high magnification (40x), a high dose of PS (PS50, 50 mg/kg) is capable of completely reversing the histological appearance of the lung SCC which resembles the bronchial epithelium layer of the VC group, a single ciliated columnar epithelium layer, as shown in Figure 2(H).

**Expression of cytokeratin 5/6 protein in the bronchial epithelium layer of the lung.**

The expression of cytokeratin 5/6 on the epithelium layer of the VC group was very low or almost non-existent, as shown in Figure 3(A). Figure 3(B) demonstrates the representative immunohistochemical staining of the NTCU group that was highly expressed with cytokeratin 5/6 protein due to a large area of brown colour staining of the bronchial epithelium layer. A weak expression of cytokeratin 5/6 was found on the bronchial epithelium layer of the mice treated with low and high doses of PS in the PS10 and PS50 groups (Figures 3C and 3D, arrow) compared to the NTCU group. Figure 4 shows the cytokeratin 5/6 quantification by measuring the percentage of pixel intensity of immunohistochemistry images using the FIJI/Image J software. A significant increase in the percentage of pixel intensity of cytokeratin 5/6 expression was observed in the NTCU group (5.20±0.43%) compared to the VC (0.98±0.10%) group (p<0.05). PS treatment significantly decreased the expression of cytokeratin 5/6, and there were significant differences in the percentage of pixel intensity of the PS10 (1.83±0.17%) and PS50 (1.25±0.19%) groups compared to the NTCU group (p<0.05). Moreover, there was no significant difference in the percentage of pixel intensity of cytokeratin 5/6 expression in the PS10 and PS50 groups compared to the VC group (p>0.05). PS50 showed a lower percentage of pixel intensity of cytokeratin 5/6 than PS10, but there was no significant difference between low and high doses of PS (p>0.05).
Histopathological scoring and the percentage numbers of mice for each group based on the carcinogenesis of lung SCC.

The H&E staining of the lung tissues of each mouse for each group was examined under a light microscope to identify the type of lesions based on carcinogenesis in the lung SCC for the score and the percentage of mice for each type of lesion in the pre-malignant stages (hyperplasia, metaplasia and dysplasia) and SCC. Dysplasia has been divided into different stages, namely low- and high-grade dysplasia, where the presence of bronchial high-grade dysplasia poses a high risk or is a precursor to the progression of lung SCC (Ishizumi et al., 2010). The dysplasia observed in our study was high-grade dysplasia with high degrees of nuclear pleomorphism, atypia was observed for the entire thickness of the bronchial epithelium layer, and the N:C ratio increased as shown in Table 1. Low-grade dysplasia can be distinguished from high-grade dysplasia with the presence of cell maturation and horizontal nuclei orientation at the upper epithelium layer and a decrease in N:C ratio (Hudish et al., 2012). Table 2 shows the histopathological score for each group with the lowest score of 0 (VC group) in which the lung histological observation of all mice samples showed a normal bronchial epithelium layer and the NTCU group had the highest score of 3.67±0.17, indicating the growth of the lung SCC. The PS10 group scored 0.67±0.12, with the majority of hyperplasia lesions was observed in the bronchial epithelium layer. The PS10 score was higher than that of the PS50 group with a score of 0.33±0.17, where the lung histological observation usually showed a normal bronchial epithelium layer. The PS10 and PS50 scores were also lower than those of the NTCU group (Table 2).

Thickness of the Bronchial Epithelium Layer

Figure 5 shows the thickness of the bronchial epithelium layer of each group. The NTCU group had the highest epithelium layer thickness (23.39±6.32µm), and a significant difference (p< 0.05) was observed in the bronchial epithelium layer thickness compared to the VC group (2.99±0.50µm). PS treatment decreased the thickness of the epithelium layer and the low dose of PS (PS10, 6.92±1.75µm) showed a significant reduction in the thickness of the bronchial epithelium layer compared to the group that received NTCU alone without PS (p< 0.05). The high
dose of PS treatment in the PS50 group (3.75±1.01µm) also showed a significant reduction in the thickness of the bronchial epithelium layer compared to the NTCU group (p<0.05). Moreover, there was no significant difference in the thickness of the bronchial epithelium layer in the VC group compared to both the low- and high-doses of the PS treatment groups, namely PS10 and PS50 (p> 0.05).

**DISCUSSION**

In this study, we revealed the potential of PS as an active natural agent that may have a chemopreventive effect on the development of lung cancer, specifically lung SCC. The development of lung SCC involves multistage carcinogenesis, in which the early stage consists of changes in the normal epithelium layer to hyperplasia followed by squamous metaplasia. The early stage progresses to the intermediate stage, where the metaplastic cells are transformed into dysplasia cells. Finally, the dysplasia cells progress and become carcinoma or cancer cells, and this process involves in situ or invasive carcinoma at the late stage of lung SCC carcinogenesis (Wang et al., 2009). Histopathological features, such as the formation of keratin pearls and intercellular bridges, increased nucleus/cytoplasm ratio and disorganisation of cells, are characteristic features for the diagnosis or differentiation of lung SCC tumours from other types of lung cancers (Franklin, 2000). The NTCU-induced lung SCC mouse model exhibits similar pathogenesis with human lung SCC compared to the histological changes involving multistage carcinogenesis mentioned above (Minna et al., 2002; Wang et al., 2009). Translation from animal studies to clinical trials is one of the challenges facing by the development of PS as an effective chemopreventive agent against lung cancer. Translational failure may occur when any interventions or drugs performed in animal studies have been shown to be successful, but the opposite results have been observed in human clinical trials. This failure may be due to disparities between the animal model and humans, such as the specificity of the disease or the ability of the animal model to accurately mimic the condition or pathogenesis of human diseases (Van der Worp et al., 2010; Mak et al., 2014). The NTCU-induced lung SCC mouse model is therefore the ideal model for testing the efficacy of any potential therapeutic or chemopreventive agent in a preclinical study to mimic this disease in humans.
The other challenge of translational failure from animal studies to the clinical trials is the conversion of the dose from animals to humans to ensure that the dose is safe and effective for humans (Nair and Jacob, 2016). Based on our findings, continuous administration of low-dose PS may be effective and safe in the prevention of the carcinogenesis of lung SCC because PS has great pharmacokinetic properties, such as long elimination half-life and good absorption (high bioavailability) (Kapetanovic et al., 2011; Wang and Sang, 2018). Treatment with a low dose of PS10 delayed early stage carcinogenesis of the lung SCC and only caused mild hyperplasia in the bronchial epithelium layer, while the high dose of PS50 may maintain a normal bronchial epithelium layer with simple ciliated columnar layer. Thus, PS can delay the development of lung SCC, as indicated by the development of lung SCC after treatment of mice with NTCU without PS. In order to further confirm the difference between the type of lung SCC lesion and other types of lung cancers, we studied the expression of cytokeratin 5/6. We found that the mice sampled treated with NTCU alone without PS were highly expressed cytokeratin 5/6 compared to the other groups. This result is supported by a study by Wang et al. (2004) in which the treatment of NTCU in the mouse lung SCC model caused an increase in the expression of cytokeratin 5/6 protein in the bronchial epithelium layer (Wang et al., 2004). In addition to the histopathological observation, the expression of cytokeratin 5/6 protein is another diagnostic biomarker for lung SCC, since the high expression of this protein acts as a specific marker for lung SCC for its differentiation from other types of lung cancers such as lung AD (Ma et al., 2015). Expression of cytokeratin 5/6 protein as a diagnostic marker for lung SCC was also demonstrated in humans, where a clinical study by Marson et al., (2004) showed that 100% of lung SCC tumours positively expressed cytokeratin 5/6. However, the expression of cytokeratin 5/6 was not detected in lung AD tumours, and its expression was less than 1% in lung LCC tumours (Marson et al., 2004). Apart from causing cell morphology changes, PS treatment also reduced the thickness of the bronchial epithelium layer. The thickness of the bronchial epithelium layer is related to the progression of the pre-malignant stages in the development of lung SCC. The bronchial epithelium layer was thickened with an increase in the severity of the pre-malignant stages in the development of the lung SCC (Dacic, 2008).

Our data from histopathological analysis and immunohistochemical staining of cytokeratin 5/6 showed an NTCU-induced lung cancer mouse model that specifically produced the lung SCC subtype. The study on a specific subtype of lung cancer is critical to the discovery of effective
chemopreventive agents because different subtypes of lung cancer exhibit a high degree of molecular heterogeneity, including the type of gene mutation (Marino et al., 2019). Chen et al., (2012) reported a chemopreventive effect against lung AD carcinogenesis following administration of PS prior to the introduction of carcinogens into the mouse (Chen et al., 2012). PS was administered prior to carcinogen therapy (NTCU) in this study to determine the chemopreventive effect of PS on lung SCC in pre-malignant stages or before the malignant stage, and this study is one of the early studies to determine the effect of PS on NTCU-induced lung SCC via the mouse model (in vivo). However, in a study on the effect of PS in the lung SCC at the malignant stage, PS was administered following the development of the lung SCC on the xenograft mouse model. The results showed that PS can significantly inhibit the growth of lung SCC tumours compared to the PS-free group (Tan et al. 2019). This finding has shown that PS can effectively stop or inhibit lung SCC if administrated either at the pre-malignant stage or after the development of lung SCC after NTCU treatment. However, further investigation is needed on the timing of PS treatment, specifically the administration of PS at the pre-malignant stages, whether during hyperplasia, metaplasia, dysplasia, or after the development of lung SCC. For instance, whether the administration of PS after the NTCU treatment at the hyperplasia stage can stop the progression of lung SCC or the administration of PS during the metaplasia or dysplasia stages is an effective chemopreventive agent against the carcinogenesis of lung SCC can be determined. Chen et al., (2012) also showed that the mechanism responsible for the chemopreventive effect of PS against the development of lung AD in the mouse model was the inhibition of the epidermal growth factor (EGFR) pathway (Chen et al., 2012). EGFR gene mutations are much more common in lung AD than in lung SCC and other lung cancers. This factor may explain why PS is targeting the EGFR pathway to prevent the development of lung AD. Different pathways may be involved in the mechanisms of PS as an anti-cancer or chemopreventive agent against a different type of lung cancer, as the prevalence of the type of gene mutations varies between lung cancer subtypes (Pikor et al., 2013; Midha et al., 2015). In lung SCC, the most common genetic abnormality is the mutation of the TP53 gene that encodes the p53 tumour suppressor protein and accounts for between 60% and 70% of the lung SCC cases (Herbst et al., 2008). Any natural agent capable of acting specifically on the p53 pathway has a great potential to be developed as an anti-cancer or chemopreventive agent against lung SCC because the p53 pathway is involved in the regulation of apoptosis and cell proliferation in carcinogenesis (Qian and Cobrinik, 2017; Aubrey et al., 2018).
Natural products with apoptotic inducer properties can be developed into drugs for cancer therapy or as chemopreventive agents, as any disruption in the apoptosis process may lead to abnormal cell growth or cancer formation (Taraphdar et al., 2001). PS can act as an apoptotic inducer in various types of cancer, including lung cancer, and it may support the chemopreventive mechanism of PS against lung SCC in this study. PS induces the activity of caspase-3 and caspase-7 as pro-apoptosis factors, leading to inhibition of the proliferation of SK-MES-1 (human lung SCC) and NCI-H460 (large cell human lung carcinoma) cell lines (Schneider et al., 2010). Caspase-3 and caspase-7 are enzymes that act as crucial mediators to induce apoptosis through a mitochondrial-dependent apoptosis pathway (Lakhami et al., 2006). Apoptosis is also responsible for the chemopreventive effect of PS against the development of lung AD by the use of the in vivo mouse model (Chen et al., 2012). Apart from being an apoptotic inducer, PS can inhibit cell proliferation, and is responsible for its anti-cancer and chemoprevention effects on various types of cancer. PS inhibits cell proliferation in various types of cancer, including hepatocellular carcinoma, melanoma, and pancreatic cancer which reduces the immunohistochemical staining of Ki-67, which is a tumour proliferation marker (Benlloch et al., 2016; Yu et al., 2019). PS treatment also increases apoptosis activity by upregulating caspase-3 expression and inhibiting cell proliferation through the down-regulation of Ki-67 expression in the immunohistochemical staining of prostate cancer (Dhar et al., 2015). Apoptosis cell death- and cell proliferation-related pathways are therefore crucial mechanisms to be studied and may be responsible for the chemopreventive effect of PS against lung SCC. Therefore, the next phase of our study will focus on the mechanisms of PS to prevent the development of lung SCC, including the apoptosis and p53 pathways. Understanding the underlying mechanisms of PS as a chemopreventive agent against lung SCC in this study is a key step in the discovery of an effective chemopreventive agent for a specific subtype of lung cancer.

In the past decades, lung cancer has been the most common type of human cancer and the leading cause of cancer-related deaths worldwide. In 2016, according to statistics published by the World Health Organisation (WHO), lung cancer caused approximately 1.7 million deaths, the highest number of deaths among all types of human cancer. Approximately 30% to 50% of all cancers are preventable by the WHO (WHO, 2018b). This study focused on the discovery of a chemopreventive agent from a natural source to prevent the development of the lung SCC. Chemoprevention of lung cancer has attracted attention as an alternative treatment to overcome
the burden of lung cancer and the potential of a natural chemopreventive agent to reduce the side effects of current drug treatment; in addition, it is cost-effective (Gullet et al., 2010). Effective chemoprevention is crucial for individuals at high risk of developing lung cancer because early stages of lung cancer are usually asymptomatic and difficult to diagnose (Birring and Peake, 2005). High-risk individuals who develop lung cancer include persons with airway obstruction, those involved in smoking and passive smoking (second-hand smoke exposure), and those with a family history (genetic susceptibility) and those living in air-contaminated areas (Kishi et al., 2002; Boffetta, 2006; Sun et al., 2017). In addition, an effective chemopreventive agent may be useful for people who have quit smoking to obtain health benefits, particularly in terms of reducing the risk of lung cancer. This action is justified by studies revealing that after smoking cessation, the risk of developing lung cancer decreases over time, but the risk of developing lung cancer remains notably higher than that of people who have never smoked even after a decade of smoking cessation (Mong et al., 2011; Tindle et al., 2018).

**CONCLUSIONS**

In conclusion, PS is a potent chemopreventive agent against the development of lung SCC as it can prevent the progression of pre-malignant stages from transforming into cancer cells or lung SCC in the mouse model.

**Abbreviations**

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Availability of data and materials
Not applicable.

Authors’ contributions
Omchit Surien, Siti Fathiah Masre, Ahmad Rohi Ghazali performed the experiment and prepared the manuscript. All authors read and approved the manuscript.

Ethics approval
In this study, the experimental animal protocols followed the approval by the Animal Ethical Committee of The National University of Malaysia (UKMAEC) with approval number FSK/2017/FATHIAH/24-MAY/846-MAY-2017-MAY-2019.

Consent for publication
Not applicable.

Competing interests
The authors declare that there is no conflict of interest regarding the publication of this article.

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REFERENCES


Tables:

Table 1: Histopathological scoring based on lung SCC carcinogenesis

<table>
<thead>
<tr>
<th>Type of lesions on the bronchial epithelium layer</th>
<th>Stages of lung SCC carcinogenesis</th>
<th>Score value</th>
<th>Image</th>
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![Image of squamous cell carcinoma](image)
Table 2: Score of histopathological scoring for each group in mean ± SEM.

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<th>Groups</th>
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<th>Stages of lung SCC carcinogenesis</th>
<th>Score value</th>
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</tr>
<tr>
<td>PS 50</td>
<td>Normal and Hyperplasia</td>
<td>Early Stage</td>
<td>0.33 ± 0.17</td>
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Figure legends:

**Figure 1:** The duration of treatment for NTCU and pterostilbene. There were two weeks of pre-treatment with pterostilbene without NTCU for the PS 10 and PS 50 groups.

**Figure 2:** The representative (haematoxylin and eosin) H & E staining of paraffin embedded lungs of mouse from each group. (A) Representative lung histology of mouse from VC group. Arrow indicates the bronchial epithelium layer. (B) Higher magnification of fig 2(A) showed the normal bronchial epithelium layer with simple ciliated columnar epithelium. (C) Representative of lung histology of mouse from NTCU group where the arrow indicates the area with thickening of bronchial epithelium layer. (D) Higher magnification of fig 2(C) showed the changes in cells with increased nucleus/cytoplasm ratio (N:C) (arrow). (E) Representative of lung histology of mice from PS 10 group showed the thickening of epithelium layer (arrow). (F) Higher magnification of fig 2(F) showed the hyperplasia (HP) (arrow) of bronchial epithelium layer. (G) Representative of histology from mouse of PS 50 group showed normal bronchial epithelium layer. (H) Higher magnification of fig 2(H) showed normal bronchial epithelium layer with single layer of ciliated columnar epithelium that resembles VC group.

**Figure 3:** Immunohistochemistry staining of cytokeratin 5/6 antibody for the representative mouse from each group. (A) Representative of immunohistochemistry staining of cytokeratin 5/6 of mouse from VC with no expression of cytokeratin 5/6, (B) The lung of mouse from NTCU group with large area and high intensity of brown staining of cytokeratin 5/6 in bronchial epithelium layer. (C) is the lung of mouse from PS 10 and (D) is the lung of mouse from PS 50,
where both have a smaller area of brown-staining. Arrows indicate the brown staining of positive area of cytokeratin 5/6 immunohistochemistry at the bronchial epithelium layer.

**Figure 4: The percentage of pixel intensity of cytokeratin 5/6 expression.** *(a) statistically significant difference between expression of cytokeratin 5/6 between VC and NTCU, (p< 0.05). *(b) statistically significant difference between expression of cytokeratin 5/6 between PS 10 and NTCU, (p< 0.05). *(c) statistically significant difference between expression of cytokeratin 5/6 between PS 50 and NTCU, (p<0.05).

**Figure 5: The thickness of the bronchial epithelium layer for each group.** *(a) statistically significant difference between the thickness of the bronchial epithelium layer of PS 10 and NTCU, (p< 0.05). *(b) statistically significant difference between thickness of the bronchial epithelium layer of PS 50 and NTCU, (p< 0.05).