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ARTICLE

SHALLOT PEEL INFUSION PREVENTS BRONCHUS EPITHELIUM THICKENING AND CILIA SHORTENING IN CIGARETTE SMOKE-INDUCED WISTAR RATS

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ABSTRACT

Cigarette smoke exposure is the considerable risk of chronic obstructive pulmonary disease, the third cause of mortality globally. Inhaled smoke triggers oxidative stress resulting in airway epithelium thickening and cilia shortening. Shallot (Allium cepa L.) peel contains flavonoids that can negate oxidative stress. The objectives of this research is to determine the correlation between shallot peel infusion (SPI), bronchus epithelium thickness, and cilia length in cigarette smoke-induced rats; and establish a maximum effective dose of SPI. Rats were divided into normal, cigarette, SPI 125, 250, 500, 1,000, and 2,000 mg/kgBW groups. Two hours after administration, rats were exposed to cigarette smoke 2 cigarettes/day for 28 days. Hematoxylin-eosin-stained bronchus was observed and variable measurements were carried out. Comparison tests of epithelium thickness and cilia length between normal and cigarette groups showed significant differences ($p<0.05$); Pearson coefficient between SPI dose and epithelium thickening was -0.614, Spearman coefficient between SPI dose and cilia length was 0.860, and maximum effective dose to prevent bronchus epithelium thickening and cilia shortening are 1,275.4 mg/kgBW and 1,325.8 mg/kgBW. In conclusion, the higher the SPI dose, the lower the epithelium thickness, and the higher the cilia length. The maximum effective dose of SPI to prevent bronchus epithelium thickening and cilia shortening are 1,275.4 mg/kgBW and 1,325.8 mg/kgBW.

Keywords: Bronchus; Cigarette; Cilia; Epithelium; Shallot; Quercetin.

АБСТРАКТ

Воздействие сигаретного дыма является основным фактором риска развития хронической обструктивной болезни легких, третьей причины смертности во всем мире. Вдыхаемый дым провоцирует окислительный стресс, приводящий к утолщению эпителия дыхательных путей и укорочению ресничек. Кожура шалота (Allium cepa L.) содержит флавоноиды, способные нейтрализовать окислительный стресс. Цели данного исследования - определить корреляцию между вливанием кожуры шалота (SPI), толщиной эпителия бронхов и длиной ресничек у крыс, подвергшихся воздействию сигаретного дыма, и установить максимальную эффективную дозу SPI. Крыс разделили на группы с нормальной дозой, дозой сигарет, дозой SPI 125, 250, 500, 1 000 и 2 000 мг/кг массы тела. Через два часа после введения препарата крыс подвергали воздействию сигаретного дыма по 2 сигареты в день в течение 28 дней. Наблюдали за бронхами, окрашенными гематоксилин-эозином, и проводили различные измерения. Сравнительные тесты толщины эпителия и длины ресничек между нормальной и сигаретной группами показали значительные различия ($p<0,05$); коэффициент Пирсона между дозой SPI и утолщением эпителия составил -0,614, коэффициент Спирмена между дозой SPI и длиной ресничек - 0,860, а максимальная эффективная доза для предотвращения утолщения эпителия бронхов и укорочения ресничек составляет 1 275,4 мг/кгБВт и 1 325,8 мг/кгБВт. В заключение следует отметить, что чем выше доза SPI, тем меньше толщина эпителия и больше длина ресничек. Максимально эффективная доза SPI для предотвращения утолщения эпителия бронхов и укорочения ресничек составляет 1 275,4 мг/кг массы тела и 1 325,8 мг/кг массы тела.

Ключевые слова: Бронх; сигарета; реснички; эпителий; шалот; кверцетин.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the third cause of mortality worldwide. According to the World Health Organization (WHO), there were 3.23 million deaths due to COPD in 2019.[1] The clinical manifestations of COPD include chronic bronchitis, emphysema, or both, which are defined by airflow limitation and persistent respiratory symptoms. The abnormality in the airway and/or alveoli caused by exposure to noxious irritant gases or particles. [2]Environmental risk factors for COPD are exposure to air pollutants from burning wood or fuel, coal dust, asbestos, silica, recurrent infections, and cigarette smoke exposure as the highest risk factor. [3,4]

Smoking is a common habit, although its hazards are well known or suffered not only by active smokers but also by passive smokers whose effects are even more harmful [5]. Inhaled cigarette smoke contains gas (92%) and particle (8%) components. The gas component consists of carbon monoxide, hydrocyanic acid, ammonia, nitrogen oxides, and formaldehyde, while the particle component includes tar, indole, nicotine, carbazole, and cresol. These particles can settle in the airway and cause airway obstruction.[6,7] Cigarette burning produces reactive oxygen species (ROS) which will provoke oxidative stress status resulting in damage and the decrease of cilia number in the airway by more than 70%.[8] A previous study demonstrated that cigarette smoke exposure 2 cigarettes/day for 28 days causes goblet cell hyperplasia. Mucus hypersecretion accompanied by bronchus cilia damage impedes mucociliary clearance. Hence, it causes bronchus epithelium thickening and bronchoconstriction.[9]

Reactive oxygen species which are very reactive free radicals need to be neutralized to cease further oxidative stress reactions. Antioxidants neutralize them one of which is by scavenging hydroxyl radicals during the peroxidation reactions of fat, protein, or other molecules in the cell membrane. They also play an essential role in avoiding increased production of proinflammatory cytokines. Flavonoid is widely sourced from natural ingredients, one of which is shallot (Allium cepa L.[10,11] Shallot peel has more flavonoids especially quercetin compared to its flesh. Quercetin compound in dry shallot peel is 20 times higher than in shallot tuber. Wiczkowski et al (2008) revealed that 83% of total quercetin in the dry shallot peel is an aglycone form which is proven to be easier to digest by the human gastrointestinal tract than its glucosides form.[12,13] The other study revealed that shallot peel extract contains 228,1 mg QE/g total flavonoid and ameliorates liver cell damage in diazinon-induced rats.[14,15]

In this study, we use shallot peel infusion (SPI) to see how it affects oxidative stress in the respiratory tract. Infusion was used to minimize the changes of quercetin derivatives before consumption. The previous study showed that quercetin contained in its food matrix has higher bioavailability than its pure and extracted form.[13,16] Flavonoid is a secondary metabolite that can dissolve in polar solvents such as water.[17] The objectives of this research are to establish the correlation between SPI dose, bronchus epithelium thickness, and cilia length in cigarette smoke-induced Wistar rats, as well as to establish the maximum effective dose of SPI to prevent bronchus epithelium thickening and cilia shortening.

MATERIAL AND METHODS

This research is true experimental with a post-test-only control group design conducted from March 2023 to June 2023 in the Pharmacology Laboratory and dan Histology Laboratory, Faculty of Medicine, University of Jember. The use of animals has followed the guide and procedures of laboratory animal caring and handling as written in the Guide for The Care and Use of Laboratory Animals Eighth Edition. We received ethical approval from the Ethics Commission of the Faculty of Medicine, University of Jember with a reference number 1.729/H25.1.11/KE/2023.

Shallot peels were submerged in 2% salt water for 10-15 minutes and subsequently washed using running water to remove dirt

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and pesticide residue and dried in the sun. The dried shallot peels were crushed using a blender and sifted to get the finest simplicia powder. The simplicia were dissolved in distilled water to prepare 20% SPI (10 g simplicia/50 mL water). The mixture was heated using a stacked pot, the first pot was filled with water which has been heated to boiling, and the second pot contained infusion solution placed on top of the first pot with a temperature of 90°C for 15 min.[18,19] The SPI was filtered using a flannel cloth and added with hot distilled water until it reached the initial volume. Serial dilution was performed to produce 10%, 5%, 2.5%, and 1.25% SPI.

The samples were male Wistar strain white rats, aged 8-10 weeks, body weight 130-180 grams. Experimental animals that undergone acclimatization for 7 days were divided into seven groups consisting of normal, cigarette, and 5 SPI groups. Shallot peel infusion groups were administered SPI at doses of 125 mg/kgBW, 250 mg/kgBW, IKBM 500 mg/kgBW, 1,000 mg/kgBW, dan 2,000 mg/kgBW orally. After 2 hours, cigarette and SPI groups were exposed to cigarette smoke 2 cigarettes/day for 28 days using a smoking chamber.

The animals were executed under ketamine and xylazine intraperitoneal anesthesia. Bronchus was washed using 0.9% NaCl and subsequently fixed in 10% Buffer Neutral Formalin (BNF). Bronchus histopathological slides were stained using Hematoxylin-Eosin (H.E.) and observed using a Leica DM500 microscope and photographed using AmScope with 400X magnification. Bronchus epithelial thickness was measured at 5 fields of view; ciliary length was measured at 4 fields of view (4 intact, straight, and clearly visible cilia per field of view).[6,20] The measurement was done using the ImageJ software on a scale of 10 µm. Before the measurement, calibration was performed to ensure the scale used was in micrometers (µm for width and length, and pixels for thickness) to fit the 400X magnification when the images were captured through the microscope. The measurement was conducted using straight trajectory lines drawn for each cilium, and with the command prompt (*ctrl+m* on the keyboard), the cilia length data (mean and standard deviation) were collected as seen in Figure 1. The mean cilia length of each group was then processed in Microsoft Excel.

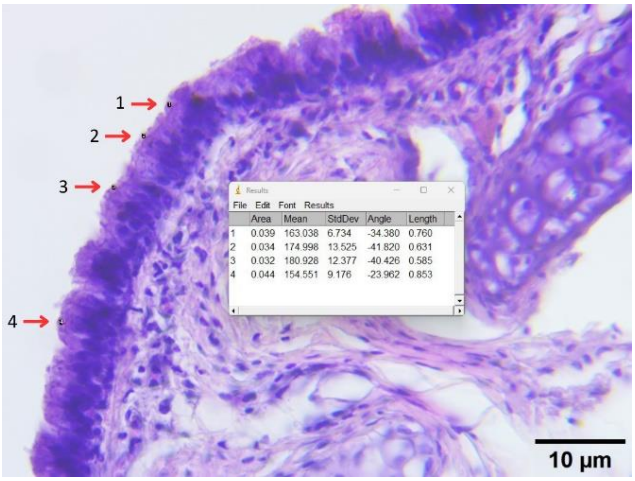

Figure 1. The measurement of cilia length using the ImageJ software on four intact, straight, and clearly visible cilia per field of view (1-4).

The data were statistically assessed using the IBM SPSS Statistics 25 application. The epithelium thickness difference between normal and cigarette groups was analyzed using the Mann-Whitney test; the cilia length difference between the normal and cigarette groups was analyzed using an independent T-test. Correlation between SPI dose, bronchus epithelium thickness, and cilia length was determined by analyzing the data from cigarette and 5 SPI groups using the Pearson test, whereas a maximum effective dose of SPI was determined using the regression test.

RESULT

The average of bronchus epithelium thickness and cilia length are presented in Table 1. The normal group shows the lowest epithelium thickness and the highest cilia length, whereas the cigarette group shows the highest epithelium thickness and the lowest cilia length.

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Table 1. The average of bronchus epithelium thickness and cilia length (µm)

Group	Bronchus epithelium thickness (µm)	Cilia length (µm)
Normal	2.636 ± 0.512	0.393 ± 0.049
Cigarette	5.515 ± 1.486	0.207 ± 0.026
SPI 125 mg/kgBW	5.007 ± 1.071	0.233 ± 0.026
SPI 250 mg/kgBW	4.368 ± 0.810	0.237 ± 0.009
SPI 500 mg/kgBW	4.092 ± 0.615	0.265 ± 0.015
SPI 1,000 mg/kgBW	3.302 ± 0.403	0.322 ± 0.029
SPI 2,000 mg/kgBW	3.895 ± 0.659	0.288 ± 0.018

Shallot peel infusion group at a dose of 125 mg/kgBW to 1,000 mg/kgBW demonstrates a decreasing trend in the value of epithelium thickness, and at a dose of 2,000 mg/kgBW demonstrates an increasing value. Bronchus epithelium histopathology is presented in Figure 2. Shallot peel infusion group at a dose of 125 mg/kgBW to 1,000 mg/kgBW demonstrates an increasing trend in the value of cilia length, and at a dose of 2,000 mg/kgBW demonstrates a decreasing value. Bronchus cilia histopathology is presented in Figure 3.

The normality test and homogeneity test were performed to analyze whether the epithelium thickness data was normally distributed and homogeneous. The normality test of epithelium thickness data resulted in a significance value of $p>0.05$ and the homogeneity test resulted in a significance value of 0.011 ($p<0.05$). Hence, the epithelium thickness difference between the normal and cigarette groups was analyzed using the Mann-Whitney test resulting in a significant value of 0.021 ($p<0.05$).

The normality test and homogeneity test were performed to analyze whether the cilia length data was normally distributed and homogeneous. The normality test of cilia length data resulted in a significance value of $p>0.05$ and the homogeneity test resulted in a significance value of 0.353 ($p>0.05$). Hence, the cilia length difference between the normal and cigarette groups was analyzed using an independent T-test resulting in a significance value of 0.001 ($p<0.05$).

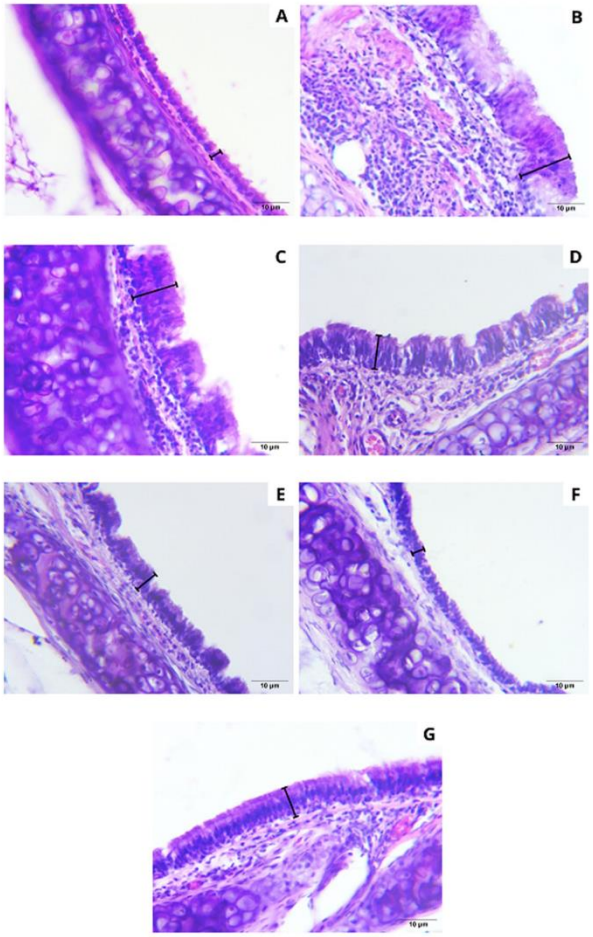

Figure 2. Bronchus epithelium histopathology. A: normal; B: cigarette; C: SPI 125 mg/kgBW; D: SPI 250 mg/kgBW; E: SPI 500 mg/kgBW; F: SPI 1,000 mg/kgBW; G: SPI 2,000 mg/kgBW. Black line: bronchus epithelium thickness.

Normality test and linearity test were performed to analyze epithelium thickness data of cigarette and 5 SPI groups. The normality test resulted in a significance value of $p>0.05$ and the linearity test resulted in a significance value of 0.619 ($p>0.05$). Pearson correlation test between SPI dose and epithelium thickness resulted in a significance value of 0.001 ($p<0.05$) and a correlation coefficient of -0.614. It indicates that there is a

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correlation between SPI dose and bronchus epithelium thickness. The higher the SPI dose, the lower the bronchus epithelium thickness in cigarette smoke-induced rats.

Normality test and linearity test were also performed to analyze cilia length data of cigarette and 5 SPI groups. The normality test resulted in a significance value of p>0.05 and the linearity test resulted in a significance value of 0.028 (p<0.05). Spearman correlation test between SPI dose and cilia length resulted in a significance value of 0.000 and a correlation coefficient of 0.860. It means that there is a correlation between SPI dose and bronchus cilia length. The higher the SPI dose, the higher the bronchus cilia length in cigarette smoke-induced rats.

The regression test for bronchus epithelium thickening showed a significance value of 0.02 (p<0.05). The quadratic curve equation obtained was:

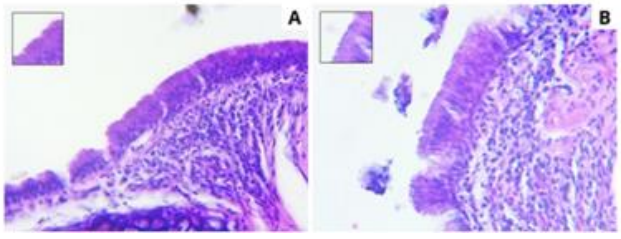
$$y=1.38 .10^{(-6)} x^2-3.52 .10^{(-3)} x+5.41 \quad (1)$$

The maximum effective dose is the x value determined using the derivative equation (y'=0). The maximum effective dose of SPI to prevent bronchus epithelium thickening is 1,275.4 mg/kgBW.

The regression test for cilia length showed a significance value of 0.00 (p<0.05). The quadratic curve equation obtained was:

$$y=0.2+1.75 .10^{(-4)} x-6.6 .10^{(-8)} x^2 \quad (2)$$

The maximum effective dose is the x value determined using the derivative equation (y'=0). The maximum effective dose of SPI to prevent bronchus epithelium thickening is 1,325.8 mg/kgBW.



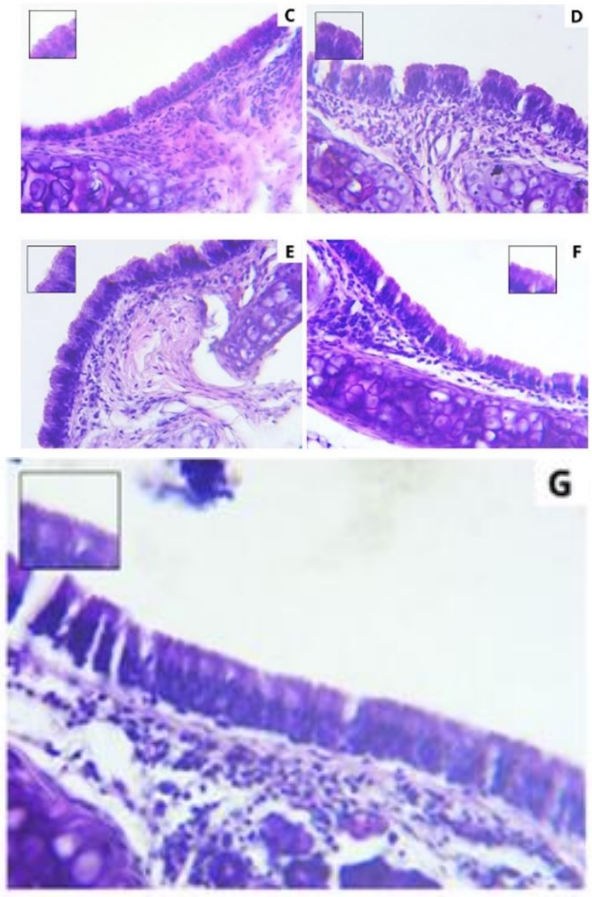


Figure 3. Bronchus cilia histopathology. A: normal; B: cigarette; C: SPI 125 mg/kgBW; D: SPI 250 mg/kgBW; E: SPI 500 mg/ kgBW; F: SPI 1,000 mg/kgBW; G: SPI 2,000 mg/kgBW.

DISCUSSION

Bronchus epithelium thickness in normal and cigarette groups showed a significant difference which means that exposure to cigarette smoke can cause bronchus epithelium thickening. A study conducted by Jeanita et al., in 2020 revealed that cigarette smoke exposure 1 cigarette/day for 14 days causes bronchus epithelium thickening [18]. Suryadinata et al. stated that cigarette smoke exposure of 2 cigarettes/day for 28 days results in the increase of bronchus goblet cell number in rat [19]. Another study demonstrated that cigarette smoke exposure of 4 cigarettes/day for 21 days causes the increase of tracheal goblet cells in rat.[20]

Bronchus epithelium is majority composed of three types of cells, i.e. goblet cells (about 30%), ciliated columnar cells

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(about 30%), and basal cells (about 30%) with other three types of cells, i.e. brush cells (about 3%), serous cells (about 3%), and cells of the diffuse neuroendocrine system (DNES) (3-4%) .[21] Bronchus epithelium thickening is one of the body responses to ROS production from cigarette burning like superoxide (O_2·), OH, nitric oxide (NO), and peroxyl ions.[22] The result of cigarette burning will also inhibit transcription factors called nuclear factor-erythroid-2 related factor 2 (Nrf-2) which decreases endogenous antioxidant gene transcription rates.[23] The increase of free radical production and the decrease of endogenous antioxidants will increase cytokines and chemokines release which will activate innate and adaptive immune cells leading to respiratory tract remodelling in the form of bronchus epithelium thickening.[6,24]

Stress oxidative condition will activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway which is followed by metalloproteinases (MMP) activity enhancement provoking tumor necrosis factor-α (TNF-α) release. TNF-α causes endothelial and neutrophil activation by increasing adhesive molecule release that induces macrophage and neutrophil infiltration to the target cells. Neutrophils move from the vascular to the tissue and secrete transforming growth factor-α (TGF-α) which afterward activate epidermal growth factor receptor (EGFR).[19,25,26] EGFR activation will induce MUC5AC gene expression resulting in bronchus epithelium thickening.[6,20,27]

The main mechanism involved in bronchus epithelium thickening is the increase of goblet cell number related to goblet cell hyperplasia.[18,20] Another mechanism associated with bronchus epithelium remodeling is metaplasia of bronchus ciliated columnar cells differentiating toward goblet cells.[20,28] In addition, bronchus epithelial progenitor cells distributed along the respiratory epithelial, i.e. basal cells also differentiate toward goblet cells.[20,21,28] Schamberger et al. revealed that cigarette smoke particularly changes the cellular structure of the respiratory tract epithelium by inducing the differentiation of basal cell.[29] Mucus hypersecretion caused by the increase of goblet cell number coincides with bronchus epithelial thickening resulting in bronchoconstriction .[30,31]

Bronchus cilia length in normal and cigarette groups showed a significant difference. It indicates that cigarette smoke exposure can cause bronchus cilia length shortening. Cilia on the surface of epithelial structures are susceptible to damage caused by exposure to irritants including cigarette smoke. A previous study explained that cigarette smoke exposure of 4 cigarettes/day for 21 days leads to a decrease in trachea cilia length in rat.[20]

Oxidative stress induced by cigarette burning causes damage to cilia protein, misfolding, ubiquitination, and intracellular aggregate protein formation. The aggregate will be recognized by histone deacetylase 6 (HDAC6) and afterward enter the lysosome to be degraded, called as autophagy.[29,32] Cigarette smoke impedes ciliogenesis by inhibiting gene expression in intraflagellar transport (IFT), cilia transcription regulation, motility, and cilia structural integrity resulting in cilia structure abnormality and the decrease of cilia length, movement frequency, and cilia coordination.[29,33,34]

A transcription factor called forkhead box protein J1 (FOXJ1) is a master regulator in ciliogenesis.[29] FOXJ1 overexpression partially inhibits cigarette smoke extract-mediated down-regulation of the basal body development gene and the IFT genes. Cigarette smoke extract alters basal cell differentiation toward secretory and ciliated cells, with metaplasia toward the squamous cell so that ciliated cells are less abundant and the cilia are shorter in those ciliated cells. The cilia length measurements from day 28 air-liquid interface (ALI) cultures showed that CSE exposure was related to shorter cilia.[35] The cilia length decrease will impede the mucociliary clearance process.[29,35]

Tubulin assembly at the distal end requires the ciliary proteins transport to the anterograde IFT at the distal end and back to

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retrograde IFT at the basal body.[36,37] A previous study demonstrated that toxic substances in cigarette smoke can reduce the genes expression involved in IFT.[38] Another study showed a total acetylated tubulin reduction in cigarette smoke exposure on primary human bronchus epithelial.[29] In addition, adenine nucleotide translocase (ANT), a protein transport of ADP/ATP located in membrane plasma mitochondria, has an important role in supporting ciliary viability by increasing oxidative respiration and ATP flux. Exposure to cigarette smoke can reduce ANT expression in the airway epithelium resulting in impaired mitochondrial respiration and ciliary viability, leading to decreased ciliary height. Conversely, the overexpression of ANT prompts airway surface hydration by ATP and supports ciliary beating post cigarette smoke exposure. [39]

A natural ingredient that can act as a source of antioxidants to prevent epithelial thickening and bronchus cilia damage is shallot.[40,41] The previous study revealed that phenolic and quercetin content in shallot peel is higher than the bulb which is commonly consumed.[42] A research conducted by Rahima et al. demonstrated total flavonoid level in shallot peel extract 228,1 mg QE/g,[14] whereas Nisak in 2020 showed total flavonoid level in *Guazuma ulmufolia* infusion was 46,2 mg QE/g .[43] In this study, an infusion of shallot peel is chosen rather than the purified extract to accommodate the aglycone forms of quercetin that make up 83% of quercetin found in the dried shallot peels. The quercetin aglycone is the free form of flavonoids that can cross the small intestine membranes barrier and thus be absorbed into the blood circulation system better.[44,45] In dried shallot peels, the quercetin aglycone is naturally dispersed in the shallot peel matrix. A previous study showed that when quercetin aglycone is provided along with their matrix as a natural source instead of its purified extract, it enhances the bioavailability thus making it very easily absorbed. The infusion method which was conducted by macerating the matrix and homogenizing the dried peels using boiled water helped to preserve the quercetin aglycone content.[16,44] Wiczkowski reported such treatment resulted in less than 5% differences in the composition of quercetin derivatives.[13] The infusion method is also said to be an effective method to isolate active compounds such as quercetin because it has the same polarity as water.[17,46] Hence, to be able to get the most quercetin content in the shallot peels, the infusion method is preferred. Shallot peel infusion is a feasible option as a functional drink because the method is easy and does not need specialized tools and techniques.

Quercetin can react with free radicals so that various ROS can be neutralized and lipid peroxidation does not occur. It can also increase the Nrf2 pathway activation thereby inhibiting NF-κB pathway and increasing antioxidant enzymes.[23] Treatment of COPD with quercetin can reduce oxidative stress conditions characterized by decreased myeloperoxidase, decreased macrophage activation, and decreased inflammatory mediator release. In addition, quercetin is also able to increase the type III protein deacetylase Sirt-1 expression which will reduce MMP9 and MMP12 activity so that progression of COPD is hampered.[23,47]

The Pearson test resulted in a correlation between SPI dose, epithelial thickness, and bronchus cilia height. The higher the SPI dose, the lower the bronchus epithelial thickness, and the higher the bronchus cilia length. It suggests that prevention of epithelial thickening and reduction of bronchus cilia length depend on SPI dose. The lowest bronchus epithelium thickness and the highest cilia length among the SPI groups are found at a dose of 1,000 mg/kgBW, while maximum effective dose of SPI to prevent bronchus epithelium thickening is 1,275.4 mg/kgBW and to prevent cilia shortening is 1,325.8 mg/kgBW. At a dose of 2,000 mg/kgBW, bronchus epithelium thickness is increased and cilia length is decreased. Thus, SPI administration exceeding the maximum effective dose causes the change of SPI property from antioxidant to pro-oxidant. High

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concentrations of antioxidants can cause cytotoxicity by inducing oxidative stress. Yang et al. revealed that quercetin and its derivates have prooxidant activity producing OH·, O2·, and H2O2 which will subsequently produce free radical compounds.[46] A research performed by Dibal et al. showed that the administration of ethyl acetate extract of shallot peel at doses of 1,600 mg/kgBW and 2,900 mg/kgBW show signs of hepatotoxicity i.e. sinusoidal dilatation, tissue hemorrhage, and lymphocyte aggregation in mice; at a dose of 5,000 mg/kgBW causes death in mice.[48] Another study conducted by Abrori et al. stated that basil leaf (*Ocimum basilicum*) extract at a dose of 2,000 mg/kg BW causes changes in renal histopathological appearance, i.e. necrosis, tubules degeneration, and proximal tubule dilatation.[49]

For further study, it is needed to perform phytochemical tests and measure the malondialdehyde (MDA) level and endogenous antioxidants such as catalase (Cat), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) to establish oxidative stress condition caused by cigarette smoke exposure. Moreover, it is also needed to identify or count the cells that makeup bronchus epithelium (ciliated columnar cells, basal cells, and goblet cells) after cigarette smoke exposure using immunohistochemistry (IHC) or stereology methods.

CONCLUSION

As conclusion, there are correlations between SPI dose, bronchus epithelium thickness, and bronchus cilia length; the higher the SPI dose, the lower epithelium thickness, the higher cilia length. Maximum effective dose of SPI to prevent bronchus epithelium thickening and cilia shortening are 1,275.4 mg/kgBW and 1,325.8 mg/kgBW.

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DECLARATIONS

Author Contributions

Concept – R.D., D.H.; Design – R.D., D.H.; Supervision – R.D., D.H.; Resources – D.H.; Materials – M.F.R.M.B., R.D.; Data Collection and/or Processing – M.F.R.M.B., R.D., E.E.; Analysis and/or Interpretation – M.F.R.M.B., R.D., E.E.; Literature Search – M.F.R.M.B., S.R.; Writing – M.F.R.M.B., R.D., E.E., D.H., S.R.; Critical Reviews – M.F.R.M.B., R.D., E.E., D.H., S.R.

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Conflict of interest

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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