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RESEARCH ARTICLE

Lung histopathological profile of male albino Wistar rats exposed to tobacco smoke administered ethanolic extract of red spinach

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Abstract

Background: Exposure to tobacco smoke, which contains free radicals, can cause oxidative stress in the lung. Oxidative stress can trigger lung inflammation due to dilation of the alveolar lumen, alveolar wall thickening, and inflammatory cellular infiltration. Red spinach (*Alternanthera amoena Voss.*) leaves have antioxidant activity in vitro, with IC50 = 4.32 µg/mL. **Objective:** To assess the antioxidant activity of red spinach in male albino Wistar rats. **Methods:** The rats were divided into five test groups of five rats each: the negative control (1% Na-CMC suspension), the positive control (vitamin C suspension), and three experimental groups receiving red spinach leaves at a dose of 200 mg/Kg BW, 400 mg/Kg BW, and 600 mg/Kg BW, respectively. All the rats were exposed to tobacco smoke for 14 days using three cigarettes in a smoking chamber. The right lung was taken for histopathology. **Results:** The results showed that doses of 200 mg/Kg BW, 400 mg/Kg BW, and 600 mg/Kg BW could reduce the level of lung damage, i.e. the enlarged alveolar lumen, thickened alveolar wall, and inflammatory cellular infiltration. **Conclusion:** The dose of 600 mg/Kg BW was effective in reducing the level of lung damage compared to the negative control.

Introduction

Tobacco use has been steadily increasing from year to year and is directly proportional to the mortality rate due to smoking. As many as eight million people die from smoking worldwide, seven million active smokers and 1.2 million passive smokers. In adults, passive smokers can develop severe respiratory diseases, in addition to coronary heart disease and lung cancer (WHO, 2019). Tobacco smoke contains thousands of toxic substances, such as free radicals, aromatic polycyclic hydrocarbons, aromatic amines, nitrosamines, and others that belong to ROS (Reactive Oxygen Species), mainly metabolised in the lungs (Fitria *et al.,* 2014). ROS trigger the immune system, including neutrophils and macrophages, thus releasing inflammatory mediators that cause inflammation in the lung cells (Lopez *et al.,* 2011). Antioxidants are compounds that can inhibit oxidation reactions by binding to free radicals and highly reactive molecules. Their function is to neutralise free radicals to protect the body from degenerative diseases (Schaal & Chellappan, 2014).

The principal function of antioxidants is to suppress the activity of ROS and the subsequent production of free radicals by inhibiting enzymes and chelating trace elements (Chen *et al.,* 2015). Flavonoids, which belong to the polyphenol group, are widely found in plants and have many bioactive effects, including anti-viral, anti-inflammatory (Wang *et al.,* 2016), cardioprotective, antidiabetic, anti-cancer (Marzouk, 2014), anti-ageing, and antioxidant properties (Munhoz *et al.,* 2014).

Red spinach (*Alternanthera amoena* Voss) is among plants that contain antioxidant compounds, i.e., flavonoids, anthocyanins, vitamin C, and tannins. It has a half-maximal inhibitory concentration (IC_{50}) of 4.32 µg/mL, indicating an excellent antioxidant activity (Syaifuddin, 2015).

Methods

Extraction

The method used was maceration; as much as 500 grams of Simplicia powder was inserted into a macerator and soaked using 96% of ethanol solvent for 24 hours in triplicates. Every 24 hours, it was filtered and replaced by a new solvent until filtrate I, II, and III were obtained. The filtrates were accommodated and concentrated using a rotary evaporator until a concentrated extract was obtained.

Experimental animals

The experimental protocol was approved by the Ethics Committee Bakti Tunas Husada Institute of Health Science No: 07/kepk-bth/04/2020. The animals were divided into five groups of five rats each: the negative control group (1% Na-CMC suspension), the positive control group (Vitamin C suspension), and three experimental groups receiving red spinach extract at the doses of 200 mg/kg BW (group 1), 400 mg/Kg BW (group 2), and 600 mg/Kg BW (group 3). All the rats were exposed to tobacco smoke for 14 days using three cigarettes in a smoking chamber. After 14 days, the experiment was terminated, and the right lungs of rats were sent to histopathology (which includes fixation, trimming, dehydration, clearing, impregnating, embedding, cutting, staining, mounting). Preparations were observed under a microscope in ten fields of view (at the four corners and the centre, with two observation points each) at a magnification of 10x more accurate results. The ImageJ software measured the targets: the alveolar lumen widening, alveolar wall thickening, and inflammatory cell infiltration.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine significant intergroup differences of each parameter. A p-value <0.05 was considered statistically significant.

Results

The Simplicia and extract contained several secondary metabolites, including alkaloids, flavonoids, polyphenols, tannins, saponins, quinones, monoterpenoids, sesquiterpenoids, steroids, and triterpenoids (Table I). The ImageJ application measured the widening of the alveolar lumen, thickening of the alveolar wall, and infiltration of inflammatory cells. The data were analysed using one-way ANOVA. Table II and Figure 1 show the results for the dilation of the alveolar lumen, thickening of alveolar walls, and infiltration of inflammatory cells.

Table I: Phytochemical screening of red spinach

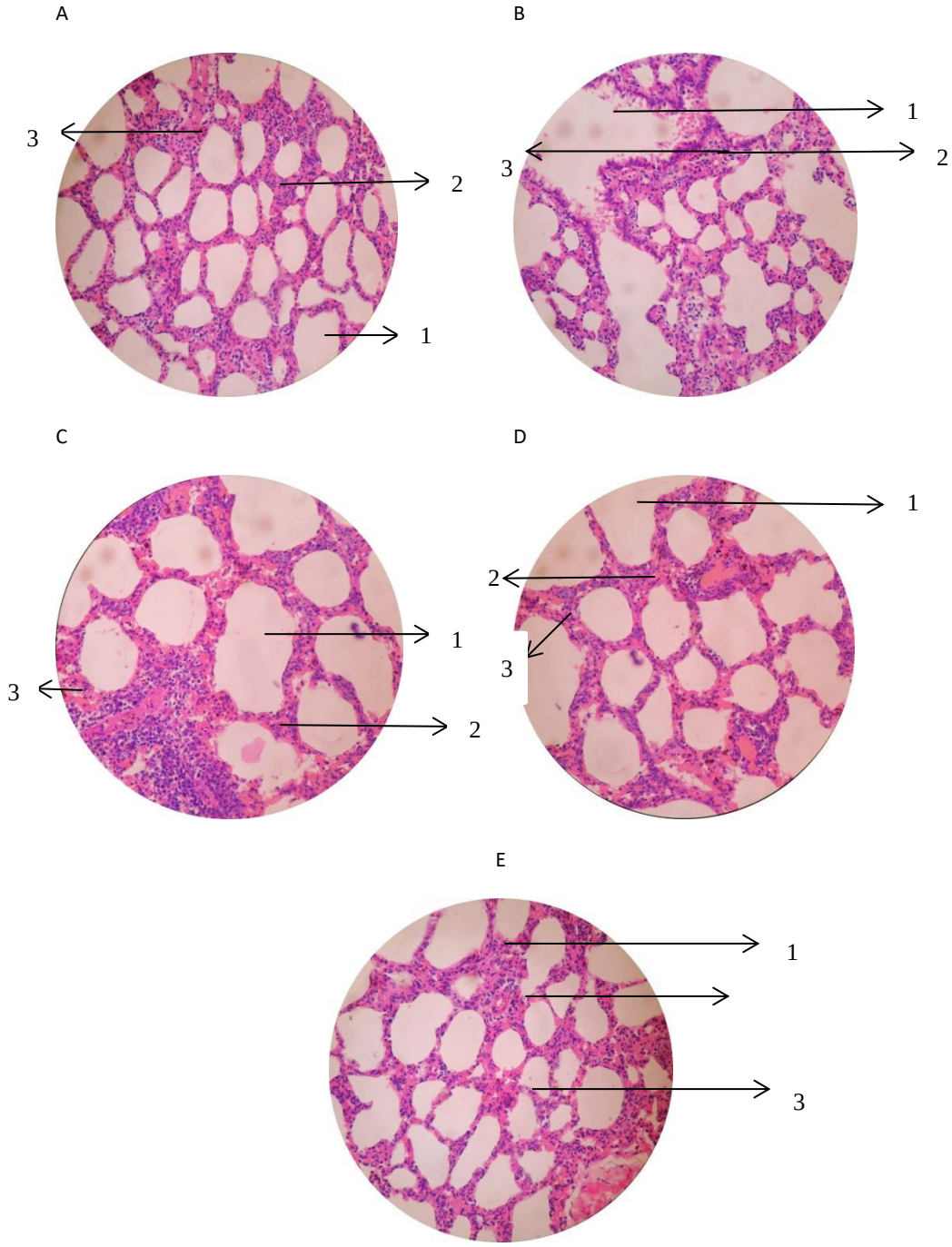
Compounds	Sample	
	Simplicia	Extract
Alkaloids	+	+
Flavonoids	+	+
Polyphenols	+	+
Tannins	+	+
Saponins	+	+
Quinones	+	+
Monoterpenoids and Sesquiterpenoids	+	+
Steroids Triterpenoids	+	+

Table II: Results of lung histopathological statistics

Group	Average histopathological result (µm)					
	Alveolar dimension	SD (±)	*p*-value	Alveolar imension	SD (±)	*p*-value
Positive Control	164.146	28.914	(0.013)	2.882	0.772	(0.049)
Negative Control	240.734	53.710	$p < 0.05$	4.642	1.429	$p < 0.05$
Dose of 200 mg/Kg BW Rat	217.262	46.050		4.404	1.409	
Dose of 400 mg/Kg BW Rat	189.073	46.507		3.804	0.885	
Dose of 600 mg/Kg BW Rat	180.007	27.763		3.256	0.591	

Discussion

The presence of alkaloids, flavonoids, tannins, saponins, quinones, steroids and triterpenoids indicated the possibility of antioxidant activity in the extract and Simplicia of red spinach leaves. The lung histopathological results were analysed based on the dilation of the alveolar lumen, thickening of the alveolar wall, and infiltration of the inflammatory cells (Bernard *et al.,* 2014). The dilation parameters of the alveolar lumen due to free radicals-containing tobacco smoke, such as NO, NO_2R, ROO^+, ROONO, superoxide, hydrogen peroxide, peroxinite, and others can induce epithelial cells to stimulate release and activation between neutrophils and macrophages.



Note: (A) Positive Control; (B) Negative Control; (C) Dose of 200 mg/Kg BW Rat; (D) Dose of 400 mg/Kg BW Rat; (E) Dose of 600 mg/Kg BW Rat; (1) Dilation of alveolar lumen; (2) Thickening of alveolar walls; (3) Infiltration of inflammatory cells. Magnification of 40 x.

Figure 1: Overview of dilation of alveolar lumen, thickening of alveolar walls and infiltration of inflammatory cells

The production of neutrophils and macrophages will inactivate anti-proteases, thus causing protease imbalances that will eventually lead to lung degradation (Ziech *et al.,* 2011). Infiltration of the inflammatory cells due to the induction of smoke-free radicals, especially neutrophils in the pulmonary blood vessels in the alveolar wall, will cause the production of inflammatory cells in the alveolus, thus triggering the thickening of the alveolar wall (Idrus *et al.,* 2016). The dilation of the alveolar lumen between the negative control group and the treatment groups had a significance of $p < 0.05$, indicating activity of lowering the size at the dilation of the alveolar lumen in the positive control group and the treatment groups. The

thickening of the alveolar wall of the positive control group had a significance of $p < 0.05$, while it was higher than 0.05 in the treatment groups because thickening of the alveolar wall occurs due to continuous infiltration of inflammatory cells due to acute exposure to tobacco smoke, making the treatment groups unable to overcome the high infiltration of inflammatory cells (Idrus *et al.,* 2016). In the absence of infiltration of inflammatory cells, the size of the normal alveolar area is 70 µm, with a thickness of 1.5 µm, larger than twice the size of the normal alveolar area and almost twice the size of the normal alveolar thickness in the positive control group (Baker *et al.,* 1979). The group with the highest dilation of the alveolus was the negative control group, due to the absence of the preparation treatment that had an antioxidant effect on the lungs and exposure to a higher level of free radicals, causing a higher level of oxidative stress than the other groups, so the negative control group had the worst damage.

Another reason is that the preparations given to the positive control were vitamin C, a powerful antioxidant. Vitamin C, which has many hydroxyl groups, will donate protons to free radicals, so the free radicals reach equilibrium, and the ascorbic acid that has given its protons will become harmless ascorbic radicals and will merge into ascorbic acid again (Muchtadi, 2009). The low dilation, especially at a dose of 600 mg/kg BW, was due to the ethanolic extract of red spinach, which contains various phytochemical compounds that can inhibit increased oxidative stress. These compounds include flavonoids, alkaloids, tannins, saponins, quinones, monoterpenoids, sesquiterpenoids, and triterpenoids.

Flavonoid compounds are thought to have antioxidant activity to lower the extent of lung damage by inhibiting the release of inflammatory cells, such as alveolar macrophages and neutrophils (Cazarolli, 2008; Lago *et al.,* 2014). Alkaloid compounds can decrease the degree of lung damage because they play a role as an anti-protease, inhibiting rhodesian and cathepsin L (Abdelmohsen *et al.,* 2012). Saponins may act as antioxidants, and they are thought to play a role as protease inhibitors that inhibit the release of cytokines, such as TNF-alpha, IL-1beta, IL-2 and interferon-gamma (Ye, X.J., & Ng, T.B. 2011). Steroid compounds and triterpenoids can act as antioxidants because they can act as protease inhibitors in trypsin activity. Observation of dilation of the alveolar lumen, thickening of the alveolar wall, and infiltration of inflammatory cells using histopathological methods usually use scoring (Tohomi *et al.,* 2014; Idrus *et al.,* 2016; Zahara & Tjiptaningrum, 2019). The plant used was a leek, resulting in a score of lung damage of 50.175±7.349 at a dose of 90mg/Kg BW (Idrus *et al.,* 2016). Another sample was savage leaf (*Premna cordifolia* Linn.), which showed the lowest percentage of lung damage at a dose of 600mg/Kg BW (Tohomi *et al.,* 2014)

Conclusion

The findings from this study show that the ethanolic extract of red spinach leaves can reduce the degree of lung damage due to exposure to tobacco smoke in male albino Wistar rats, the effective dose being 600 mg/kg BW.

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