



THE EFFECT OF GARLIC (*ALLIUM SATIVUM*) EXTRACT ON THE HISTOPATOLOGICAL LUNG OF BALB/C MICE WITH NICOTINE EXPOSURE

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ABSTRACT

Background: Nicotine is known to cause damage to the lungs via oxidative stress that triggers an inflammatory response, thus damaging the alveoli. Garlic extract can act as an antioxidant that counter the oxidative stress caused by nicotine exposure. **Objective:** This study is to proving the effect of garlic extract on lung histopathology of BALB/c mice by giving two dosage regiments orally and exposed the mice to nicotine by inhalation. **Methods:** The study used an experimental posttest-only control group design which was carried in for 24 days. Mice were given an adaptation period of 7 days followed by a treatment period of 14 days. On the last day, the lungs was taken for histopathological tests. Mice were divided into 4 groups, namely a negative control group as group 1, a positive control group as group 2, treatment group 1 as group 3, and treatment group 2 as group 4. Histopathological preparations were made on each sample stained with Hematoxylin-Eosin paint which was then observed microscopically and assessed for severity of damage using scoring criteria. **Results:** The degree of lung damage were scored 0, mild, moderate, and severe respectively. Group 1 : 73%,27%,0%,0%; Group 2 : 0%,0%,33%,67%; Group 3 : 0%,33%,67%,0%; Group 4 : 0%,100%,0%,0%. **Conclusion:** The effect of garlic significantly show in group 4.

Keywords: Nicotin, Garlic, Lung Damage

INTRODUCTION

Smoking is one of the main risk factors attributing to diseases of the respiratory system.¹ According to data from basic health research (Riskesmas) conducted by the Ministry of Health of the Republic of Indonesia, there is an increase in the prevalence of smoking in Indonesia every year, especially in the population aged 15-24 years.² In 2013, 7.2% of the Indonesian population aged 10-18 years became active smokers. In 2018, the results of the Riskesdas research stated that there was an increase in active smokers as much as 1.9% of the population aged 10-18 years.³ The World Health Organization states that every year, smoking kills eight million people, the United Nations reports that every year 3.3 million people die from problems in the respiratory system due to smoking. 60,000 children under the age of five die from infections that occur in the lungs due to passive smoking.⁴

Nicotiana tobacum, and Nicotiana rustica are one of the main raw materials for processed cigarettes.⁵ Conventional cigarettes or processed tobacco cigarettes are the most widely consumed types of cigarettes among the public.⁶ Based on the data obtained, the number of conventional smokers in Indonesia ranks third in the world after China and

India.⁷ Around 4000 chemical substances contained in conventional cigarette smoke are harmful to health, including carbon monoxide, nitrosamines, and tar. According to researches, these substances have the potential to be dangerous if consumed continuously on the body, including lung tissue.⁶

Electric cigarettes have the main task of delivering nicotine vapors to smokers.⁸ Electric cigarettes have an increasing popularity among the public, especially those of the productive age, especially in western countries.⁹ Many people think that electric cigarettes are safer than conventional cigarettes. However this is not true, because the danger lies in what kind of liquid is vaporized by the cigarette.¹⁰

Previous research has stated that every exhaled cigarette smoke contains 4-6 times more nicotine than what is inhaled by the user, in other words passive smokers are more at risk than active smokers, this is because the amount of harmful substances inhaled by passive smokers is higher.¹¹ This results in the high incidence of diseases in the respiratory system caused by smoking.¹² Based on data, in 2001 9.8% of Indonesian population deaths due to smoking were caused by chronic lung disease and emphysema. Exposure of nicotine in children



can make their lung growth getting slower and it's make children more susceptible to respiratory tract infections and exacerbates asthma.^{13,14} It is estimated that in 2030 deaths from smoking will reach 10 million per year and in developing countries no less than 70% of deaths due to smoking.¹⁵ The increasing number of smoking-related deaths is directly proportional to the number of adolescent smokers who increase every year.^{1,14,16}

Nicotine is contained in tobacco leaves, which can provide some good psychological stimulation effects on the body, such as increased concentration, changes of mood, and sensations of pleasure. However, if consumed excessively, it can cause structural damage to the lungs.¹⁷ Burning liquid cigarettes will produce toxic substances such as propylene glycerol, glycerin, nicotine, acetaldehyde, formaldehyde, and acrolein.^{16,18,19} This nicotine content acts as an prooxidant or free radicals that can trigger inflammation to cause cancer.²⁰ Active free radicals can attack cellular components of the body such as lipid compounds, carbohydrates, and proteins that will cause damage to both cell structure and function.²¹ The effect is not only caused by nicotine, other substances such as propylene glycol are using for the solvent and the flavour also taking part cause the lungs damage.²²

As one of the excretory organs, the lungs have the role to excrete substances that are not needed by the body.²³ The toxic effects that can emerge are degeneration, necrosis, and thickening of the alveolar septa.²⁴ Massive nicotine exposure can cause damage to the lungs of mice with a moderate degree of damage.²⁵

Some studies say that garlic has a protective effect on the incidence of respiratory system diseases. Garlic has moderate antioxidant content.²⁶ Garlic extract contains minerals, fatty acids, antioxidant compounds, and antibacterial compounds.^{27,28} The content of organosulfur and flavonoid compounds acts as antioxidants are useful as they promote balance between oxidants and antioxidants.²⁹ Daniela et al stated that garlic has high antioxidant and anticancer properties and has been proven microscopically.³⁰

Based on these problems, researchers are interested in finding out whether the administration of two doses garlic extract would affect the histopathological features of lungs that are exposed to nicotine.

METHODOLOGY

The study used a true experimental research design with a post-test control group research design which was carried out for 4 months from April to July 2021. The extract was made at the Terpadu Laboratory of Diponegoro University. The research conducted in the Biology Laboratory of Semarang State University.

On the 15th day, before making lung preparations, mice will be anesthetized. After the mice are under anesthesia, the lungs are taken. Histopathological test was performed with HE staining. Lung tissue fixed in 10% formalin.

Inclusion criteria: BALB/c mice, male, weight 25-30 grams, age 6-8 weeks, and healthy. Drop out criteria: experimental animals died during the study. The stratified dose of garlic extract used was 300 mg/KgBW and 500 mg/KgBW given orally and the nicotine dose used was 10 ml/KgBW exposed by inhalation.

The study consisted of 4 experimental groups with each group consisting of 6 BALB/c mice which were previously adapted for 7 days and randomized, the treatment was carried out for 14 days, with details:

- 1) Group 1 (C-) A negative control group. The group that was only given standard feed for 14 days.
- 2) Group 2 (C+) A positive control group. The group that will be given nicotine exposure at a dose of 10 ml/KgBW/day by inhalation for 14 days.
- 3) Group 3 (T1) The group of mice exposed to nicotine at a dose of 10 ml/KgBW/day by inhalation and given garlic extract at a dose of 300 mg/kgBW/day via gastric probe for 14 days simultaneously.
- 4) Group 4 (T2) The group of mice exposed to nicotine at a dose of 10 ml/KgBW/day by inhalation and given garlic extract at a dose of 500 mg/kgBW/day via gastric probe for 14 days simultaneously.

The preparations were read in 5 fields of view with a magnification of 400x. Scoring is used to assess the degree of lung damage in table 1 from Hansel and Barnes.

The results of the data were analyzed with the Social Sciences Statistical Package software (SPSS). Normality test was carried out and the data distribution was not normal. Subsequently, the

hypothesis was tested using the Kruskal Wallis test which was then followed by the Mann-Whitney test. The data will be considered meaningful if the results are $p < 0.05$.

Table 1. Degree of Lung Damage Hansel and Barnes

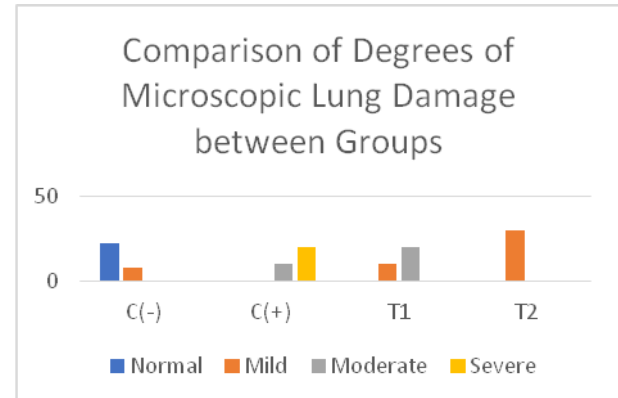
Criteria ^{25,31}	Description	Score
Normal	No damage found	0
Mild Damage	There are signs of damage such as inflammatory cell infiltration or pulmonary edema or damage to lung constituent cells <30% of the visual field.	1
Moderate Damage	There are signs of damage such as inflammatory cell infiltration or pulmonary edema or damage to cells that make up the lung 30%-60% of the visual field	2
Severe Damage	There are signs of damage such as inflammatory cell infiltration or pulmonary edema or damage to lung constituent cells >60% of visual field	3

This research has obtained ethical clearance from the Medical and Health Research Ethics Commission, Faculty of Medicine, University of Diponegoro / Central General Hospital dr. Kariadi, Semarang with ethical clearance number 49/EC/H/FK-UNDIP/V/2021.

RESULT

At the time of the study, no drop out criteria were found. The histopathological picture assessment uses the percentage of damage degrees from Hansel and Barnes. Degrees of Lung Damage, which are divided into 4 scoring levels, namely normal levels, mild damage degrees <30% visual field, moderate damage 30-60% visual field, and severe damage >

60% field of view.²⁵ Here are the results of his research:



- 1) Group 1 (C-) Group 1 had normal lung microscopy where 73% showed normal readings and 27% samples had mild damage.
- 2) Group 2 (C+) Group 2 most of the samples were found to have severe damage as much as 67% and moderate damage as much as 33%. The majority of samples in the K(+) group had severe emphysema and inflammatory cell infiltration between the pneumocytes.
- 3) Group 3 (T1) In group 3, 67% of the samples had moderate damage and 33% of the samples had mild damage.
- 4) Group 4 (T2) Obtained 100% of the samples with mild damage.

Histopathological description of the lungs of BALB/c mice at 400x magnification is presented in the following figure:

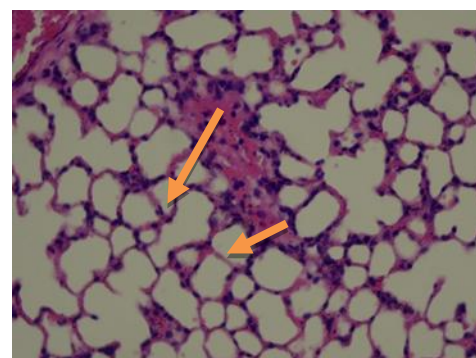


Figure 1. Normal pulmonary histopathology

At 400x magnification found normal alveolar shape. The septum in the alveolus is visible (orange arrow). No inflammatory cells were found in the interstitial cells of the pneumocytes.

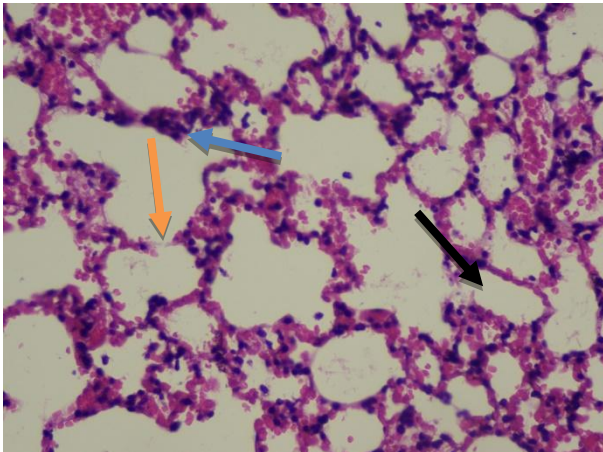


Figure 2. Lung histopathology mild damage

At 400x magnification, it was found that the shape of the alveolus began to widen and several fused alveoli were found (black arrow). The septum in the alveolus begins to break down. Septum alveolus starting damage (orange arrow). The presence of inflammatory cells in the interstitial cells of pneumocytes were minimal/relatively clean (blue arrows). Pneumocytic cell damage < 30% field of view.

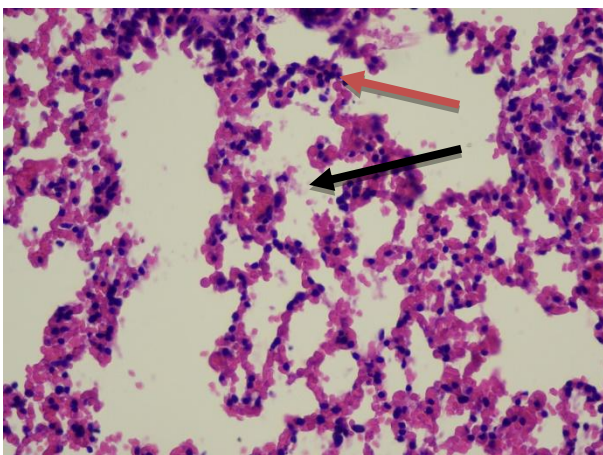


Figure 3. Lung histopathology moderate damage

At 400x magnification, it was found that the shape of the alveolus had begun to widen and several septums in the alveolus had begun to be damaged so that there was fusion between the alveoli (black arrows). More inflammatory cells were found in the

interstitial space (red arrow). Pneumocytic cell damage 30%-60% of the visual field.

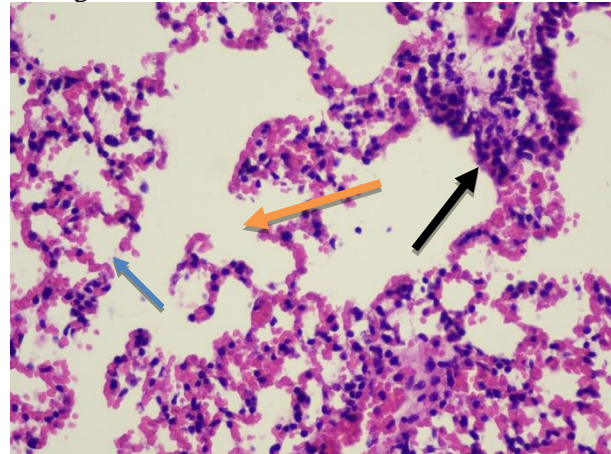


Figure 4. Lung histopathology severe damage

At 400x magnification, the alveoli are dilated and fused. Fused alveoli (orange arrow). Alveoli are filled with inflammatory cells in large numbers (black arrow). Damage septum alveoli (blue arrow). Pneumocytic cell damage > 60% visual field.

In the statistical test, it was found that the distribution of data was not normal in the Normality Test, then the Kruskal-Wallis test was carried out to see the differences in all groups. From the test, obtained p value <0.05, which means there is a significant difference. Furthermore, the Mann – Whitney test was carried out to see the comparison between the 2 groups. Significant results obtained p <0.05.

Table 2. Results of Mann-Whitney

	CH	C-	T1	T2
C-	–	<0,001*	<0,001*	<0,001*
C+	<0,001*	–	<0,001*	<0,001*
T1	<0,001*	<0,001*	–	<0,001*
T2	<0,001*	<0,001*	<0,001*	–

Note : * Significant (p < 0,05)

DISCUSSION

Free radicals that arise from the exposure of nicotine can cause oxidative stress, which then triggers the activation of inflammation that will lead to the recruitment of inflammatory cells. Prostaglandin is one of the pro-inflammatory mediators that are synthesized from arachidonic acid with the help of cyclooxygenase enzymes (COX1 and COX2) which then cause inflammation, necrosis, changes in the shape of the alveolar septum and



edema. Garlic, as an antioxidant-rich ingredient, plays a role in protecting and maintaining cells.⁶

Most of the samples from the Group 1 showed normal histopathological features, but there were also mild damage in some samples. From the observation of 30 visual fields, 22 fields of view were found to be included in the normal degree and 8 fields of view were included in the degree of mild damage. In preparations with mild damage in this group, there were several lungs that had emphysema and minimal lymphocyte and macrophage cells were seen. This is due to the absence of exposure to toxic substances such as formaldehyde, carbon monoxide, benzene, and others. This also happened in a study conducted by Putra et al, which showed that the group not exposed to nicotine showed normal pulmonary alveoli.⁶

The effect of nicotine exposure on the lungs can be seen in the Group 2 (C+). The real effect of nicotine exposure can be proven with this group. From 30 fields of view, 20 fields of view are classified as severe degrees and 10 fields of view are moderate degrees. This is in accordance with research in the form of a literature review that has been carried out previously by Sumbayak et al., stated in their journal that nicotine exposure causes damage to the lungs as evidenced in some experimental-based research literature on mice and rats exposed to inhaled nicotine.³² In this Positive Control Group, microscopic observations showed more damaged alveolar septum, wider alveolus diameter than the other groups, and most of the lumen was covered with inflammatory cells. The mice in this study can be assumed to be like passive smokers. Through exposure to burning nicotine, toxic substances enter the body through the respiratory system and will affect the organs of the respiratory system, especially the alveoli. Continuous exposure to nicotine can cause more severe effects such as lung cancer. Research conducted by Tang et al on FVB/N rats with a nicotine exposure model by inhalation with a dose larger than this study, namely 36 mg for 4 hours per day, 5 days per week, and carried out in a treatment period of 54 weeks, the results obtained 9 out of 40 mice in the nicotine-exposed group had lung adenocarcinoma.³³

The group 3 on microscopic observation of 30 fields of view obtained 20 fields of view showing moderate damage and 10 fields of view showing mild damage. Group 3 obtained a microscopic

picture of a score of 1 and a score of 2. In 10 fields of view, it described the presence of dilated and fused alveoli as a picture of pulmonary emphysema. The alveolar septum was found to be damaged and some inflammatory cells were found, but the microscopic picture was still relatively clean. In 20 visual fields, the septum was seen more damaged, there were alveolus that widened in diameter, at the moderate degree of damage, there were more inflammatory cells compared to the mild degree of damage. Inflammatory cell count at damage score of 2 was included in moderate. The visual field is still normal about 40%. The administration of garlic extract in a dose of 300 mg/KgBW/day resulted in a significant effect when compared to the Group 2. This can also be seen in the data analysis which states that there are significant results. The content of organosulfur and flavonoids which act as antioxidant compounds as an antidote to toxic substances from burning nicotine. The anti-cancer, anti-inflammatory, and anti-oxidant activities of the compounds contained in garlic such as allicin, quercetin glycosides, S-allyl-L-cysteines (SAC), diallil sulfida (DAS), diallil disulfida (DADS), diallil trisulfida (DATS), and S-allilmerkaptosistein (SAMC) have been proven to be a solution to the dangers of nicotine exposure. These chemicals made the outcome of lung samples better when compared to Group 2, in which the majority had moderate to severe damage and an inflammatory cell infiltrate that filled the interstitial lung cells. When compared with the Group 1, group 3 had a microscopic picture that was not as good as the results of the Group 1 sample. However, the results are more effective and have a greater therapeutic value shown in experiments with a dose of 500 mg/KgBW garlic extract.

Group 4 obtained microscopically the degree of lung damage was mild.. From 30 fields of view, all readings were obtained with a mild degree of damage. The picture of inflammatory cell infiltration is minimal or can be said to be relatively clean, and there is little alveolar damage such as dilated and fused alveoli. This shows the good effectiveness of the use of garlic extract as a solution for exposure to cigarette smoke, especially in passive smokers. The content of organosulfur and flavonoid compounds in garlic has been shown to be able to act as an antidote to free radicals caused by inhalation of nicotine exposure. Comparison of the treatment group that received 500 mg/KgBW garlic extract with the group



1 and group 2 had significant results. Therefore, the active content of garlic was able to prevent the occurrence of severe lung damage as in the Group 2 which showed inflammation results in pneumocytes and the presence of inflammatory cells in the moderate to severe degree. The results of this study were also obtained by Daniela et al using experimental mice induced by acrylamide and given garlic extract at a dose of 500 mg/KgBB proving that garlic extract has anticancer activity and has proven it microscopically.³⁰ The use of garlic extract as a solution to cigarette smoke exposure needs to be continuously studied to get better sample outcomes.

Microscopic observations in the 2 treatment groups that each received a dose of garlic 300 mg/KgBW/day and 500 mg/KgBW/day showed that in each group there was still damage. However, when compared between the two groups, the group receiving 500 mg/KgBW/day had less severe alveolar damage compared to the group receiving 300 mg/KgBW/day garlic extract. Microscopic observation of the lung alveolus of mice that had been exposed to nicotine by inhalation in 5 microscopic fields of view found that there was alveolar damage that occurred in the group 2, and the treatment group, both group 3 and group 4. Based on statistical data analysis, there are significant differences in the comparison between groups. In this study, onion extract at a dose of 500 mg/KgBW/day showed the best effectiveness compared to an extract dose of 300 mg/KgBW/day and was able to show a therapeutic effect on lung cell damage caused by inhalation of nicotine exposure. This is due to the organosulfur and flavonoid compounds from garlic which have anti-inflammatory and anti-oxidant functions that can inhibit the delivery of pro-inflammatory signals such as nitric oxide and prostaglandins.^{21,30}

This study is in line with a previous research conducted by Herdiani et al. who found that inhalation exposure to nicotine can damage the lungs of wistar rats because of the toxic substances contained in cigarette smoke and can get worse when exposed to continuously and at higher doses. Toxic substances produced from burning nicotine can easily increase the percentage of lung pneumocyte cell damage so that it can cause changes in lung structure and physiological function.³⁴

In each group, exposure to nicotine 10 mg/KgBW/day by inhalation had shown damage to

the lungs and none showed lung adenocarcinoma in the sample. This may be related to the duration of nicotine exposure and the level of dose administered to the mice. Previous research that is in line with this study was conducted by Eva Tyas Utami et al entitled "Effect of Exposure to E-Cigarette Smoke on Lung Histology of Balb/c Male Mice (*Mus musculus* L.)" where there is an effect of graded doses of nicotine on tissue structure lungs. In this study, inhaled nicotine exposure doses of 1 ml, 2 ml, and 4 ml were administered for 4 weeks. At a dose of 4 ml, there was destruction of the septa and widening of the alveoli. Exposure to nicotine by inhalation is precise and can describe the original condition in life.³⁵

This study has several similarities with the research conducted by Daniela et al entitled "Effectiveness of Sulfide Compounds in Garlic Against the Risk of Lung Cancer". In this study, Daniela et al. used mice as experimental animals which were induced by acrylamide and given a graded dose of garlic extract. The study used 4 groups, where the treatment group was given multilevel doses of 500 mg/KgBW/day and 1000 mg/KgBW/day. At this dose, it has been shown to have good effectiveness as a solution for exposure to cigarette smoke.³⁰

CONCLUSION

Group 4 shown better result from the histopathological than group 3. That's mean that garlic extract having therapeutic effect in dose 500 mg/KgBW/day than 300 mg/KgBW/day as antioxidant to protect the cells from nicotine as prooxidant.

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