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ANTIOXIDANT EFFECTIVENESS OF ASTAXANTHIN BY ASSESSING GLUTATHION (GSH) CONDITIONS OF WISTAR RATS (RATTUS NORVEGICUS) EXPOSED TO CIGARETTE SMOKE

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Abstract

This study aims to see if exposure to cigarette smoke as a source of free radicals can reduce glutathione levels and also to see the antioxidant effect of astaxanthin as a free radical antidote. This research design was carried out using a laboratory experimental method with a post-test control group design with a sample size of 20 experimental animals divided into 4 groups, namely K- (ordinary air + distilled water), K+ (2 cigarettes + distilled water), KP1 (2 cigarettes cigarettes + astaxanthin 12 mg/rat/day) and KP2 (5 cigarettes + astaxathin 12 mg/rat/day. This research was conducted at the Integrated Laboratory, Faculty of Veterinary Medicine, UNHAS, Hasanuddin University Medical Research Center (HUM-RC) Hospital Laboratory Hasanuddin University Makassar. The results showed that serum GSH levels were p=0.096 (p>0.05); lung histopathological checking was p=0.001 (p<0.05). It was concluded that there was no significant difference between GSH levels serum after treatment among all groups, there were significant differences in lung inflammation score data in the research groups.

Keywords: Astaxanthin, Glutathion, Antioxidant, Cigarette Smoke.

INTRODUCTION

Cigarette smoke contains a complex mixture of toxins, some of those toxins are free radicals. Cigarette smoke can be broken down into gas and particulates, each form has different chemical substances. Overall, the gas form makes oxidation while the particulate form become reduction [1].

Based on data from the World Health Organization (WHO), there are 1.3 billion smokers in the world and third of them come from the global population aged 15 years and over. The smoking habit can cause health problems. Smoking also not only harms yourself but also harms other people around you [2]. In Indonesia, around 65.6 million women and 43 million children are exposed to cigarette smoke or become passive smokers. Many Indonesian are exposed to cigarette smoke because 91.8% of smokers smoke at home [3]. One of the main sources of free radicals is cigarette smoke. The dangers of smoking not only haunt those who are active smokers, but also spread to passive smokers. The possibility of passive smokers experiencing health problems due to inhaled cigarette smoke reaches 30% [4].

Giving additional antioxidant in conditions of oxidative stress will help body to fight oxidant molecules that come from inside and outside of body, so it helps to reduce or prevent oxidative damage [5], [6]. Glutathione (GSH) is a tripeptide that composed of the amino acids glutamate (Glu), cysteine (Cys), glycine (Gly). As an antioxidant, glutathione chemically react with singlet oxygen, superoxide radicals, hydroxyl, and directly act as a free radical scavenger. Glutathione can also stabilize membrane structures by eliminating or minimizing the formation of peroxides in lipid peroxidation reactions [7].

One of exogenous antioxidant is astaxanthin, a carotenoid that is found in many marine organisms. Astaxanthin is a potent antioxidant with antioxidant power 100 times stronger than α-tocopherol and 40 times stronger than ß-carotene. However, unlike other carotenoids, astaxanthin does not show character of prooxidant [8].This research was conducted with purpose of seeing whether exposure to cigarette smoke is a source of free radicals which able to reduce glutathione levels and also aims to see the antioxidant effect of astaxanthin as an antidote to free radicals.

MATERIALS AND METHODS

Location and Research Design

This research was carried out at the Integrated Laboratory, Faculty of Veterinary Medicine, Hasanuddin University Makassar to carry out maintenance and treatment of experimental animals and at the Hasanuddin University Medical Research Center (HUM-RC) Laboratory, Hospital of Hasanuddin State University Makassar to check serum of GSH using the ELISA method with a kit insert BT Lab. The design of this research is laboratory experimental research with a post-test control group design.

Research Sample

The sample in this study was male Wistar rats (Rattus norvegicus) which kept and bred in the animal laboratory, Faculty of Medical Hasanuddin University Makassar with aged 2-3 months and weighed 150-200 grams, 20 mice were divided into 4 groups, namely the control group. Negative (K-), positive control group (K+), treatment group 1 (KP-1) with 12 mg astaxanthin, treatment group 2 (KP-2) with 12 mg astaxanthin. The inclusion criteria for the sample were healthy, active, 2-3 months old, male, weight >200 grams. The exclusion criteria for the sample were weight <150 grams, hair loss, anatomical abnormalities, illness or experiencing health problems during adaptation period.

Adaptation of Experimental Animals

Before the experiment, the experimental animals were adapted in a cage for 7 days to make their life and food are same. The health of the experimental animals was monitored every day and weighed every week. After adaptation, the mice were placed individually in their own cages and given food and drink water ad libitum, keeping the cage environment so that it was not humid, controlling the room temperature and providing sufficient light.

Astaxanthin Intervention

After the adaptation period ends, the experimental animals will be divided into 4 groups randomly with same number, namely n=5 in each group. The negative control group (K-) was the group exposed to normal air + distilled water at a dose of 5 ml/each mouse

(morning and afternoon) for 28 days. The positive control group (K+) was the group that exposed 2 cigarettes every day (morning and afternoon) + distilled water ad libitum (morning and afternoon) for 28 days. Treatment group 1 (KP-1) is group that exposed cigarette smoke with 2 cigarettes a day (morning and afternoon) + astaxanthin 12 mg/rat/day for 28 days. Treatment group 2 (KP-2) is group that exposed cigarette smoke with 5 cigarettes per day (morning and afternoon) + astaxanthin 12 mg/rat/day for 28 days. After the 29th day, all treatment groups had taken their blood and then their lungs were dissected.

Exposure of Cigarette Smoke

Exposure of cigarette smoke in this study used a chamber box. Exposure of cigarette smoke was giving cigarette smoke in the rat cage to expose the rat with cigarette smoke. One cigarette will be finished in 15 minutes. The cigarettes used in this study were Sampoerna Hijau which contained 38 mg TAR and 2.3 mg nicotine per stick.

Serum GSH Examination

Blood samples of 3 mL of experimental animals were centrifuged for 15 minutes at speed of 2000 rpm. After centrifugation, 200 µl of serum was pipetted using a micropipette into an Eppendorf tube, the serum samples that had been obtained then checking using the GSH ELISA Kit (BT Lab, China) according to the manufacturer's instructions. Optical density values were read using a microplate reader with a wavelength of 450 nm.

Lung Histopathological Examination

The lung organ was carried out part checking using hematoxylin and eosin (H&E) staining, which will then be checked under a microscope with 100x and 400x zoom. The procedure of measuring the degree of lung inflammation is based on research by Ning et al. (2013), where the degree of lung inflammation was evaluated using a subjective scale ranging from 0 to 4 by observing inflammatory cell infiltration and obstruction of the bronchiolar lumen by mucus and cell debris. The interpretation of the lung inflammation degree score is 0 = normal, 1 = mild inflammation, 2 = moderate inflammation, 3 = severe inflammation, and 4 = very heavy inflammation.

Statistic Anaylisis

Normality test used the Shapiro-Wilk test. The data is normally distributed with a p value > 0.05. Homogeneity test using Levene's Test. Homogeneous data with p value > 0.05. Comparative Test, the analysis used to test the difference of two unpaired group variables (control group and treatment group) is the unpaired T-test with a significance level of 5% ($p > 0.05$) if the data is normally distributed. In addition, a type of comparability test analysis using Mann-Whitney will be used if the data is not normally distributed.

Research Ethics

Research ethics after approval by the Health Research Ethics Commission (KEPK) Hasanuddin University Medical Faculty-UNHAS State University Hospital (RSPTN UH), with Letter Number 101/UN4.6.4.5.31/ PP36/ 2022.

RESULT

Serum GSH Levels

Previously, the research data was tested for normality of GSH level data, the results obtained were that the GSH level data before and after treatment were normally distributed with values of p=0.081 and p=0.666 (p>0.05).

Next, a comparative test of GSH data was carried out using Oneway ANOVA test and the result was p = 0.096 (p>0.05), which means there was no significant difference in GSH levels after treatment among all groups. The results of checking GSH levels after 28 days of treatment in each research group can be seen in Picture 1.

From Picture 1 it is shown that average GSH levels of group mice exposed to cigarette smoke, namely K+, KP1 and KP2, were lower than average GSH levels of the K- group which were not exposed to cigarette smoke.

The lowest average GSH level was shown by the K2 group (5 cigarettes/day + cigarette smoke) with a value of 2,54.89 + 78.67 ng/mL. Based on statistical results, there were no differences in GSH levels among groups after treatment.

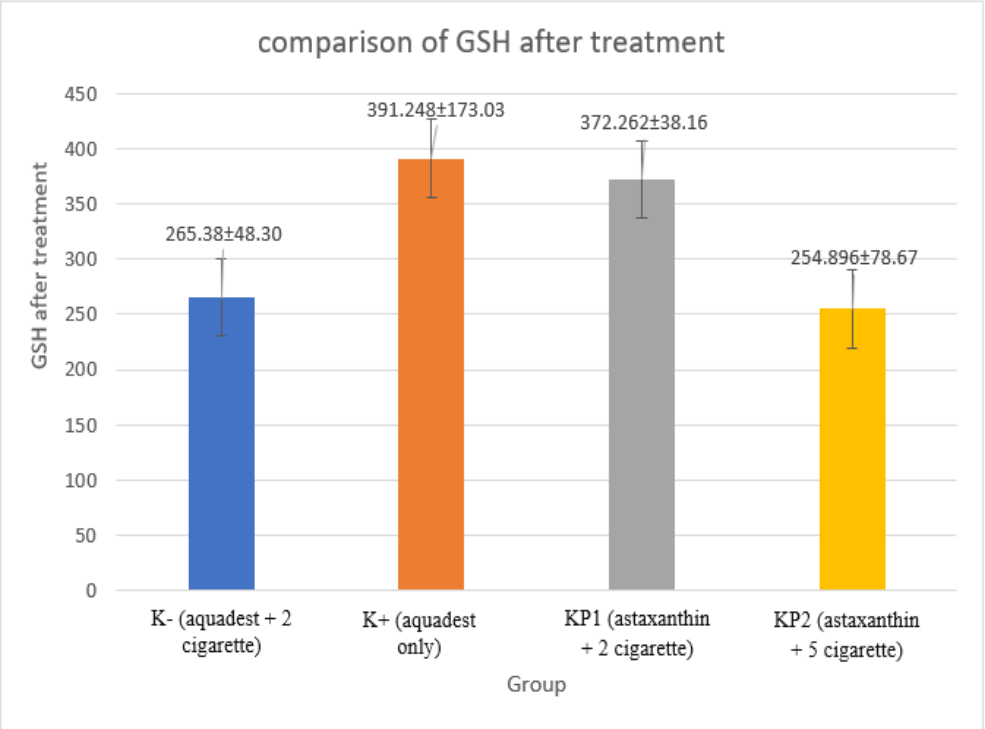

Figure 1: Diagram of Mean Serum GSH levels for Each Study Group. Data are Presented as Mean + SD

To assess differences in GSH before and after treatment, a paired T test was used because the data were normally distributed. Statistical results found that there were significant differences between GSH levels before and after treatment, which can be seen in Table 1.

Table 1: Differences in GSH levels before and after treatment

Paired Samples Test								
	Paired Differences					t	df	Sig. (2tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
GSH_BEFORE - GSH_AFTER	81.63950	175.01941	39.13553	-.27210	163.55110	2.086	19	.051

Based on statistical results, it was found that the value of p = 0.051 (p > 0.05) was not significant but was clinically significant with a value of t = 2.086 (t > 2) = significant.

Lung Organ Histopathology

Histopathological checking of lung part is carried out to assess the degree of lung inflammation. The normality test for inflammation score data in the research group showed that the data was not normally distributed with a value of p=0.000 (p<0.05). So that the comparative test used was the Kruskal-Wallis test where the result was p=0.001 (p<0.05) which indicated that there was a significant difference in lung inflammation score data in research group. Next, the Mann-Whitney test was continued to know the significance of each group. The results of the average inflammation score from histopathological checking of mice lung part after 28 days of treatment can be seen in Picture 2.

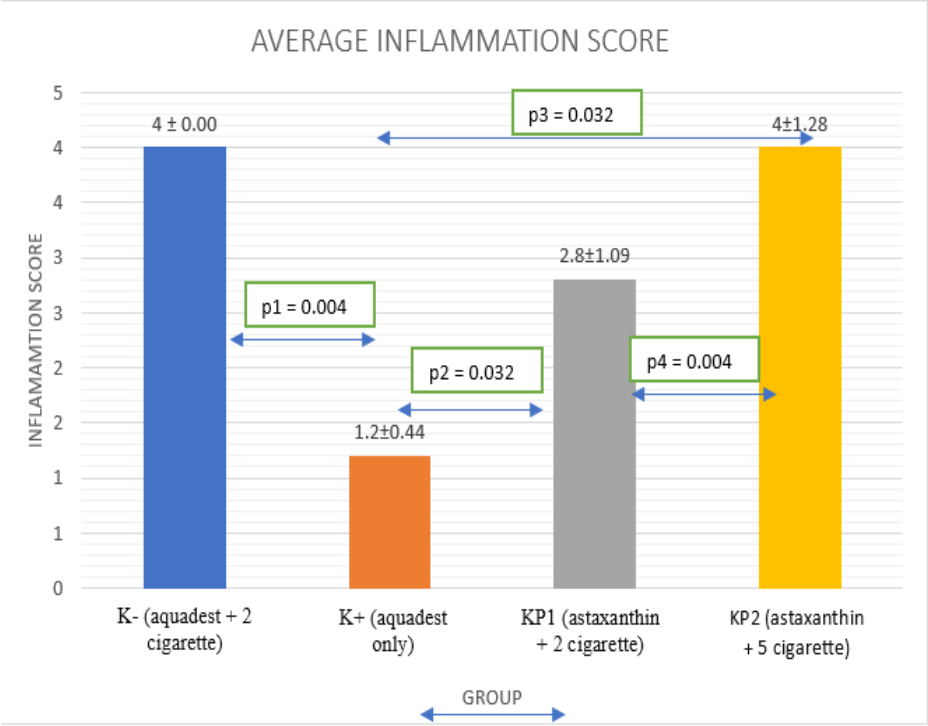

Figure 2: Diagram of Mean Inflammation Score from lung histopathology examination. Data are presented as Mean + SD

From Picture 2 above, it can be concluded that the highest average inflammation score was obtained from the KP2 group of 4+1.28 who were exposed to cigarette smoke of 5 cigarettes/day and astaxanthin intervention for 28 days, and the average value was significantly different compared with the average inflammation score in the K- group which was not exposed to cigarette smoke.

Table 2: Comparison test K- and K+ group

Ranks				
Rat Treatment	**N**	**Mean Rank**	**Sum of Ranks**	**p-value**
Aquadest	5	8	40	
Aquadest + 2 cigarette	5	3	15	0,004
Total	10			

Based on the comparison test between the K- and K+ groups using the Mann-Whitney test, it can be seen in Table 2. The value obtained was p=0.004 (p<0.005), which means there is a significant difference in inflammation score between the K- groups (aquades + no cigarette smoke) with K+ (aquadest + 2 cigarettes/day).

Table 3: Comparison test K+ and KP1 group

Ranks				
Rat Treatment	**N**	**Mean Rank**	**Sum of Ranks**	**p-value**
Aquadest + 2 Cigarette	5	3.60	18.00	
2 Cigarette + Astaxanthin	5	7.40	37.00	0,032
Total	10			

Based on the comparison test between the K+ and KP1 groups using the Mann-Whitney test, it can be seen in Table 3. The value obtained was p=0.032 (p<0.005), which means there is a significant difference in inflammation score between the K+ groups (aquadest + 2 cigarettes/day) with KP1 (2 cigarettes/day + astaxanthin).

Table 4: Comparison test K+ and KP2 group

Ranks				
Rat Treatment	**N**	**Mean Rank**	**Sum of Ranks**	**p-value**
Aquadest + 2 Cigarette	5	3.00	15.00	
5 Cigarette + Astaxanthin	5	8.00	40.00	0,004
Total	10			

Based on the comparison test between the K+ and KP2 groups using the Mann-Whitney test, it can be seen in Table 4. The value obtained was p=0.004 (p<0.005), which means there is a significant difference in inflammation score between the K+ groups (aquadest + 2 cigarettes/day) with KP2 (5 cigarettes/day + astaxanthin).

Table 5: Comparison test KP1 and KP2 group

Ranks				
Rat Treatment	**N**	**Mean Rank**	**Sum of Ranks**	**p-value**
2 Cigarette + Astaxanthin	5	3.50	17.50	
5 Cigarette + Astaxanthin	5	7.50	37.50	0,017
Total	10			

Based on the comparison test between the KP1 and KP2 groups using the Mann-Whitney test, it can be seen in Table 5. The value of p=0.017 (p<0.005) was obtained, which means there is a significant difference in inflammation score between the KP1 groups (2 cigarettes/day + astaxanthin) with KP2 (5 cigarettes/day + astaxanthin).

The histopathological image of mouse lung with H&E staining after treatment for 28 days can be seen in Picture 3.

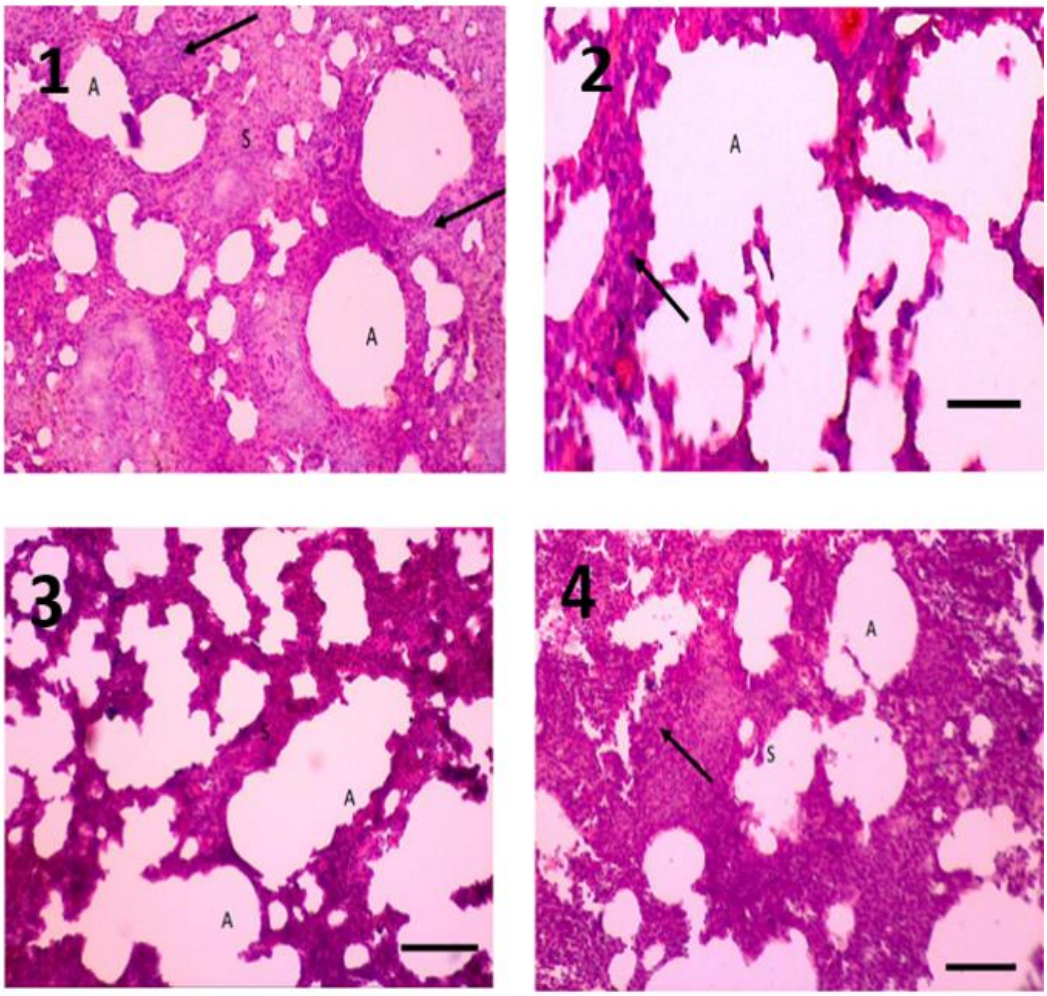

Figure 3: Histopathology results, inflammatory cell infiltration (black arrow); A (Alveol), S (Interalveolar septa), HE 10x. 1) Lung Histopathology K+ high degree of inflammation; 2) Lung histopathology K- mild degree of inflammation; 3) Lung histopathology KP1 low degree of inflammation; 4) Lung histopathology KP2 severe degree of inflammation

DISCUSSION

Based on the statistical results of serum GSH levels, there were no differences in serum GSH levels among groups after treatment. However, statistical results found that there was a significant difference between GSH levels before and after treatment. The results of serum GSH levels in the KP1 group showed a decrease in GSH before and after treatment, while for K+ and KP2 there was an increase in serum GSH before and after treatment.

This research is in accordance with previous research conducted by Alkadrie, A.T. (2017) conducted research on the effect of administering astaxanthin on glutathione levels and found that average GSH levels with a dose of 12 mg/day were lower than the neutral control and negative control groups, while the dose of 24 mg/day and the dose of 48 mg/day had higher values of GSH level was higher compared to the negative control.

Figure 3 shows the histopathological results in the K+ group who were treated with 2 cigarettes of cigarette smoke per day and distilled water for 28 days. There was very heavy infiltration of alveolar inflammatory cells and interalveolar septa, thus receiving an inflammation score of 4 (very severe inflammation). In figure 2) shows the results of histopathological examination in the K- group which was treated only with distilled water for 28 days, showing mild inflammatory cell infiltration of the alveoli and interalveolar septa, thus receiving an inflammation score of 1 (mild inflammation). Figure 3) shows the results of the histology examination on sample KP1, showing heavy infiltration of alveolar inflammatory cells and interalveolar septa, thus receiving an inflammation score of 3 (severe inflammation). Figure 4 shows the results of the histology examination on sample KP2, showing very heavy infiltration of alveolar inflammatory cells and interalveolar septa, thus receiving an inflammation score of 4 (very haevy inflammation).

Based on histopathology results, it showed that giving dose of astaxanthin 12 mg/day had a lower degree of lung damage in mice exposed to 2 cigarettes of cigarette smoke per day compared to the group of mice exposed to 2 cigarettes of cigarette smoke per day with distilled water. This shows that the higher the dose of astaxanthin given, the higher the effectiveness in improving the degree of lung damage in mice exposed to cigarette smoke.

Research by Akduman, H., et al, suggested that astaxanthin has been proven to reduce lung damage caused by inflammation and hyperoxia with anti-inflammatory, anti-oxidant, anti-apoptotic properties, and protects the lungs from heavy damage [9].

Astaxanthin protect oxidative stress through Nrf2 and improves emphysema caused by cigarette smoke. Therapy with astaxanthin directed at activating the Nrf2 pathway has the potential to be a new preventive and therapeutic strategy for COPD [10].

Astaxanthin reduced levels of oxidative stress, as demonstrated by reduced plasma malondialdehyde levels, and reversed age-related changes in residual skin surface components in middle-aged subjects [11]. When given as a supplement to type 2 diabetes mellitus patients, it improves serum lipid profiles, increases adiponectin levels, and lowers blood pressure [12]. Mortality and histological damage due to acute lung injury improved with astaxanthin treatment and reduced oxidative stress and inflammatory responses [13].

Limitation in this research is procedure for handling experimental animals, as well as laboratory environmental conditions which can also potentially cause inflammation in the respiratory tract in experimental animals.

CONCLUSION

There was a difference in serum GSH levels before and after treatment. However, there was no difference in serum GSH levels among treatment groups. There was a significant difference in inflammation score between the K- and K+ groups, there was a difference in inflammation score between the K+ and KP1 groups, there was a difference in inflammation score between the K+ and KP2 groups, there was a difference in inflammation score between the KP1 and KP2 groups.

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Conflict of Interest

There is no conflict interest

References

1) Effendi K. and Dwitiyanti, “Pengaruh Pemberian Ekstrak Etanol 70% daun Bayam Merah (Amaranthu tricolor L) Dalam Menghambat Kecacatan Fetus Mencit Bunting yang Terpapar Asap Rokok. ,” Universitas Muhammadiyah Prof. Dr. Hamka, Jakarta, 2020.

2) Alviventiasari, R. Ismail, S.A. and Susilaningsih, N. “Pengaruh Pemberian Dosis Bertingkat Jus Mengkudu (Morinda citrifolia L.) Terhadap Jumlah Eritrosit Tikus Galur Wistar (Rattus norvegicus) Yang Diberi Paparan Asap Rokok,” Universitas Diponegoro, Semarang, 2012.

3) Hanum, H. and Wibowo, A. “Pengaruh Paparan Asap Rokok Lingkungan Pada Ibu Hamil Terhadap Kejadian Berat Bayi Lahir Rendah,” *Majority*, pp. 22–26, 2016.

4) Suryadinata, V., Bambang, W., and Merryana, A. “Pengaruh Perubahan Hiperlansia Sel Goblet Selama 28 hari Paparan Asap Rokok Dengan Pemberian Antioksidan Superoxide Dismutase,” *Indones. J. Public Heal.*, vol. 11, no. 1, pp. 60–68, Dec. 2016.

5) Duthie, G. and Bellizzi, M. “Effects of Antioxidant on Vascular Heath,” *Br. Med. Bull*, vol. 55, no. 3, pp. 568–577, 1999.

6) Menvielle-Bourg, F. “Superoxide dismutase (SOD), a Powerfull Antioxidant, is Now Available Orally,” *Phylotherapie*, vol. 3, pp. 1–4, 2005.

7) Winarsi, H. *Antioksidan Alami dan Radikal Bebas*. Yogyakarta: Penerbit Kanisius, 2007.

8) Alkadrie, A. T. “Pengaruh Pemberian Astaxanthin terhadap Kadar Glutation pada Kerusakan Jaringan Testis Tikus Putih (Rattus norvegicus) Jantan Galur Wistar yang Diinduksi Formaldehid Secara Oral,” Universitas Tanjungpura, Pontianak, 2017.

9) Akduman, H. *et al.*, “Astaxanthin Reduces the Severity of Intestinal Damage in a Neonatal Rat Model of Necrotizing Enterocolitis,” *Am. J. Perinatol.*, vol. 39, no. 16, pp. 1820–1827, Dec. 2022.

10) Hiroaki, K., Kazuhisa, A., Kazuya, K., Arata, S., Yohkoh, K., and Atsuko, O. “Astaxanthin Supresses Cigarette Smoke-Induced Emphysema through Nrf2 Activation in Mice,” *Mar. Drugs*, vol. 17, no. 12, p. 673, 2019.

11) Chalyk, N.E., Klochkov, V.A., Bandaletova, T.Y., Kyle, N.H. and Petyaev, I.M. “Continuous astaxanthin intake reduces oxidative stress and reverses age-related morphological changes of residual skin surface components in middle-aged volunteers,” *Nutr. Res.*, vol. 48, pp. 40–48, Dec. 2017.

12) Zakerkish, M. “Astaxanthin improves glulcose metabolism and reduces blood pressure in patients with type 2 diabetes mellitus,” *Pac. J. Clin. Nutr*, vol. 27, pp. 341–346, 2018.

13) Bi, J., Cui, R., Li, Z., Liu, C. and Zhang, J. “Astaxanthin alleviated acute lung injury by inhibiting oxidative/nitrative stress and the inflammatory response in mice.,” *Biomed. Pharmacother.*, vol. 95, pp. 974–982, 2017.