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Acute and Subchronic Toxicity of Indonesian House Dust Mites (IHDM) Allergenic Extract for Asthma Allergy Immunotherapy

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Abstract

Background*: In developing a pharmaceutical product, it is necessary to conduct pre-clinical and clinical trials to ensure its safety and effectiveness. The toxicity test is conducted to assess the safety of a substance to determine its toxic effect of the substance.* ***Objective****: This study aims to determine the acute and subchronic toxicity of administering IHDM allergenic extract using experimental animal models.* ***Methods****: Female BALB/c mice and female and male Wistar rats were used as experimental animal models. While the IHDM allergenic extract was used with the level of Der p1 is 11.3-26.6 ng/mL and was administered by intravenous route. The acute toxicity test was carried out for 14 days on four different dose groups of experimental animals. The subchronic toxicity test was carried out for 28 days using three other dose groups of experimental animals.* ***Results****: The administration of a single dose of IHDM allergenic extract at various doses did not cause mice behaviour changes, and no death was shown in each group. Likewise, there was no change in the principal organs by macroscopic observations. Meanwhile, administering IHDM allergenic extract at repeated doses for 28 days could show signs of toxicity. The symptoms were shown in the histopathological structure of the liver, kidney, and heart organs.* ***Conclusion****: It can be concluded that the IHDM allergenic extract is safe for single-dose administration but shows toxic signs when given in repeated doses. Further tests are needed for 90 days of subchronic toxicity and satellite testing.*

Keywords*: acute toxicity, subchronic toxicity, IHDM safety, asthma allergy, neglected disease*

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INTRODUCTION

Asthma is a chronic lung disease that has affected approximately 300 million people worldwide, and this disease causes about 346,000 deaths annually (Sisson et al., 2018). According to the Global Burden of Disease, it was estimated that 420,000 people died from asthma, and there were more than 1,000 deaths per day in 2016 (GAN, 2018). This disease can attack everyone and is one of the leading causes of early dying and can also cause a decrease in the quality of life in all populations (GAN, 2018). Asthma is estimated to affect about 339 million people worldwide. Asthma is one of the leading causes of disability and mortality for sufferers (GAN, 2018).

Asthma is generally characterized by chronic inflammation of the airways. The respiratory symptoms are wheezing, shortness of breath, chest tightness, shortness of breath, and cough that varies periodically with persistently restricted expiratory airflow. The symptoms can arise due to several causes, such as sporting activities, exposure to allergens or irritants, changes in weather, or viral infection of the respiratory tract. These symptoms may disappear spontaneously during the time, but symptoms of asthma exacerbation may also threaten the sufferer's life (GINA, 2020).

Allergic asthma involves cell inflammation, including eosinophils, mast cells, T lymphocytes, neutrophils, and macrophages. There is a bias in the immune response in asthma, which includes the infiltration of Helper 2 T cells into the lungs and the presence of secretions cytokines (IL-4, IL-5, IL-9, IL-13, and IL-33). This cytokine is a sign of eosinophil inflammation, leading to the production of specific IgE allergens and the presence of airway hyperresponsiveness (AHR), and the release of inflammatory mediators such as eosinophils, mast cells, T and lymphocytes, neutrophils (Balkrishna *et al.*, 2020).

House Dust Mites (HDM) is one of the most common causes of respiratory allergies globally. It can be found in children, adolescents, and adults—high exposure to mite allergens. Asthma patients sensitive to HDM can trigger bronchospasm and increased bronchial hyperreactivity. At the same time, cessation of exposure to allergens can relieve these symptoms (Zuiani & Custovic, 2020). *Dermatophagoides pteronyssinus* (Der p) has at least 23 allergens predicted to contribute to the sensitization process through proteolytic activity, activating the body's innate immune cells underlies the type 2 adaptive immune response (Hesse *et al.*, 2020). Currently, allergen immunotherapy is one of the treatments for allergic asthma. Allergen immunotherapy is a treatment strategy for IgE-mediated allergic disease. According to Drazdauskaitė *et al.* (2021), allergen immunotherapy is the only therapy that can modify the immunological processes underlying specific allergic asthma to immunotherapy. In recent years, it has been recognized that immunotherapy of HDM allergenic extract has been widely registered as a pharmaceutical product in allergic asthma (Eguiluz-gracia *et al.*, 2020).

In Indonesia, an HDM allergenic extract product was developed and is used as immunotherapy. In creating a pharmaceutical product, it is necessary to conduct pre-clinical and clinical trials to ensure its safety and effectiveness of a pharmaceutical product. Pre-clinical testing is carried out through two test stages, namely: effectiveness test and toxicity test. The effectiveness test is carried out to determine the effectiveness of a compound or substance. In contrast, the toxicity test is carried out to assess the safety of a compound or substance to determine the toxic effect of a compound or substance. Several studies found that allergen extracts had different allergenicity when they came from various regions and/or were developed with other manufacturers (Cheong *et al.*, 2009; Zimmer, Vieths & Kaul, 2016).

This is evidenced by several studies showing that the administration of HDM allergens with various variations causes differences in T cell responses, binding to IgE, and even the toxicity effect which may occur due to: differences in amino acid sequences in allergenic proteins, differences in raw materials, and differences in composition (Hales *et al.*, 2002; Weghofer *et al.*, 2008; Casset *et al.*, 2012). Therefore, this study will examine acute and subchronic toxicity in the desensitization of IHDM allergenic extract.

MATERIALS AND METHODS

Materials

Allergenic extract

The allergenic extract used is Indonesian house dust mites allergenic extract (Der p1 = 11.3-26.6 ng/mL). The allergenic extract was purchased from Dr Soetomo Regional Hospital (Surabaya, Indonesia). The allergenic extract was administrated by intravenous injection.

Experimental animals

Mice and rats were purchased from Pusat Veterener Farma, Ministry of Agriculture of The Republic of Indonesia (Surabaya, Indonesia). Healthy female

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BALB/c strain mice aged 6-8 with 20-25 g in weight, which required nulliparous non-pregnant, were used for the acute toxicity test. Healthy male and female Wistar strain rats aged 6-8 weeks with 250-300 grams. All experimental animals were adapted for at least a week. All animals were housed in ventilated cages with ad libitum access to water, a standard pelleted diet under controlled temperature conditions (23±2 oC), and a light cycle (12 hours light/dark). All animal procedures were carried out in compliance with the ethical committee of the Faculty of Veterinary Medicine, Universitas Airlangga, for the use of laboratory animals (Number: 2.KE.058.05.2021).

Method

Acute toxicity test

The acute toxicity test was done as the Guideline of Preclinical Toxicity Test from the National Agency for Food and Drug Control of The Republic of Indonesia and the Organisation for Economic Co-Operation and Development (OECD) Guideline. In the acute toxicity test, mice were divided into four groups, namely: the control group were given saline. Group 1 was given the Indonesian HDM with a dose of 0.13 mg. Group II was given the Indonesian HDM with a dose of 1.3 mg. Group III was given the Indonesian HDM with a dose of 2.6 mg. Each group consisted of 10 mice. The observation was done for the first 30 minutes, 4 hours, and 24 hours for 14 days. The mice were sacrificed on the 15th day.

Subchronic toxicity test

The subchronic toxicity test was done as the Guideline of Preclinical Toxicity Test from the National Agency for Food and Drug Control of The Republic of Indonesia and the OECD Guideline. In the subchronic toxicity test, rats were divided into three groups: the Control group were given saline. The Indonesian HDM was given to Group 1 at a dose of 0.09 mg. The Indonesian HDM was given to Group II at a dose of 0.9 mg. The observation lasted 28 days. On the 29th day, the mice were sacrificed.

Parameter

Bodyweight observation

In the acute toxicity test, the body weight of the mice was observed for 14 days. In the subchronic toxicity test, the body weight of the rats was observed for 28 days.

Behavioural observation

Changes in behaviour that can be visually observed are walking backwards or walking in circles and changes in the eyes, skin, and feet. In addition, it is also observed the presence of symptoms of toxicity that arise in the body of rats, such as the emergence of spots, sores, nasal discharge, and even death (Ningsih *et al.*, 2017).

Organs' weight and gross pathology observation

Animal organs (heart, liver, and kidney) were weighed and measured the size using millimetre block for the toxicity tests after the animals were sacrificed. The presence of changes in shape, colour, or size and visible lesions were also observed.

Histopathological observation

In the subchronic toxicity test, organs taken from animals are then fixed in a 10% formalin solution for one week. Then paraffin blocks are made and cut into 4 mm. After that, Haematoxylin & Eosin staining was performed on the specimens and observations were made using a microscope at 400 times magnification. Each organ was scored for lesions and then compared between the group.

LD50 Calculation

LD50 calculation is done using linear regression probit analysis with the help of the IBM SPSS Vers. 26 Software (New York, USA).

Table 1. The organs' histopathological toxicity scoring

Organs	Description	Toxicity Score
Heart	Normal cell condition	1
	Mild lesion	2
	Moderate lesion	3
	Severe lesion	4
Liver	Normal cell condition	1
	Portal inflammation	2
	Degeneration parenchymatous or fibrotic	3
	Presence of necrosis	4
Kidney (presence of lesions)	< 25%	1
	26 – 50 %	2
	51 – 75 %	3
	76 – 100 %	4

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Statistical data analysis

Data of bodyweight was statistically analyzed using Two-Way ANOVA. Data of the organs' weight and histopathological toxicity score were statistically analyzed using One-Way ANOVA. All statistical data processing was processed using the Graph Pad Prism 9.0 program (California, USA).

RESULTS AND DISCUSSION

Acute toxicity test

Bodyweight observation

The weighing of the mice was carried out every day. In the acute toxicity test, the body weight of mice was weighed every day for 14 days and then recorded for later analysis of changes in daily body weight in each group of experimental animals. Figure 1 shows the weight profile of mice in the acute test toxicity were weighed daily for 14 days. This picture shows no change or significant daily weight loss (Two-Way ANOVA, F(13.504)=1.191, P=0.2820). As for the dose group 3 at days 1-3 seen weight loss, but on the 4th day and subsequent weight gain. In the control group, doses 1 and 2 showed no change in weight that stands out daily.

Behavioral observation

No toxicity symptoms and death occurred during the test, so the probit analysis was not done.

Organs' weight and gross pathology observation

The heart, liver, and kidney were weighed and observed for the presence of changes in shape, color, or size and visible lesions of each group's organ.

In Figure 2 above, it can be seen that the liver size between the Control group, Group 1, Group 2, and Group 3, have been the same between groups, and does not appear differences in the colour of the liver organs between each group. Each animal's heart size group in the heart organ is known to have almost the same size between groups. In the kidney organs, it is known that there were no visible changes in colour between organs of each group, and visual size visually looks no different in each group.

It is known in Figure 3. It indicates no significant difference in organs' weight between each group (One-Way ANOVA, F(3,24)=2.236, P=0.1099 for the liver; F(3,24)=3.386, P=0.0214 for the heart; F(3,24)=1.750, P = 0.1836 for the kidney).

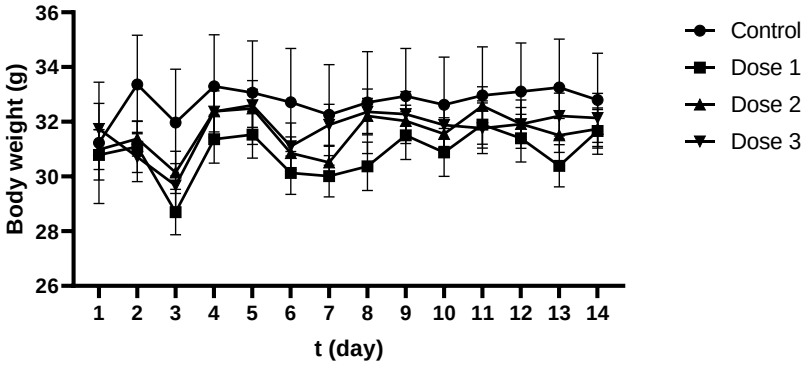

Figure 1. Bodyweight changes of mice used for acute toxicity test

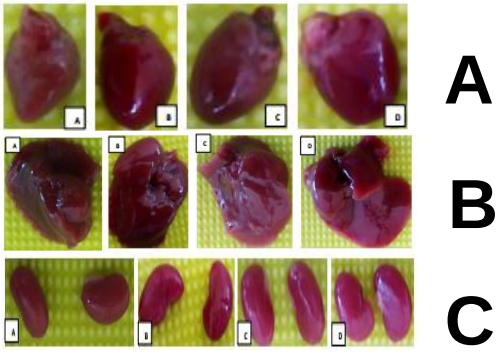

Figure 2. The visual appearance of the heart (A), liver (B), and kidney (C) organs for each group. (a) Control group; (b) Group I; (c) Group II; and (d) Group III

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Subchronic toxicity test

Bodyweight observation

The weighing of the rats was carried out every day for 28 days. Figure 4 shows a graphic profile of rats' body weight in each group in the subchronic toxicity test weighed daily for 28 days. The data obtained showed no significant change in body weight from each group each day (Two-Way Anova F(27,336)=0.5829 P=0.9540 for males; F(27,336)=0,2229 P>0.9999 for females).

Behavioural observation

In the subchronic toxicity test, two rats died during the 28-day observation period, a rat in Group I and a rat in Group II. These data indicate that IHDM allergenic extract administration repeatedly for 28 days can cause toxicity in experimental animals starting at a dose of 0.09 mg.

Organs' weight and gross pathology observation

The heart, liver, and kidney were weighed and observed for changes in shape, color, or size and visible lesions of each group's organ. In Figure 5, there appears to be no size difference between the Control group, Group I, and Group II in the rat liver. Then, there are no differences in colour, size, and shape for the heart organ when compared. In the kidney, the differences can be observed in each group. In the Control group, the colour is reddish-brown, Group 1 is reddish-brown but more faded, and Group 2 looks dark brown. These observations show that giving repeated-dose IHDM allergenic extracts can cause toxic effects on kidneys were observed changes visually in kidney color.

Based on the graph above, it was seen that there was no significant change in organs' weight between each group (One-Way Anova; F(2.6)=3.429, P=0.1016 for the liver; F(2.6)=0.3750, P=0.7023 for the heart; F(2,6)=0,2523, P=0,7849 for the kidney).

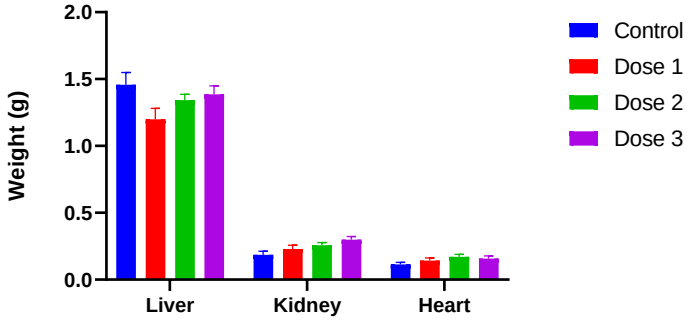

Figure 3. Each group's organs' weight profile of heart, liver, and kidney. Data are means ± SEM (n=10) *P<0.05 compared to control

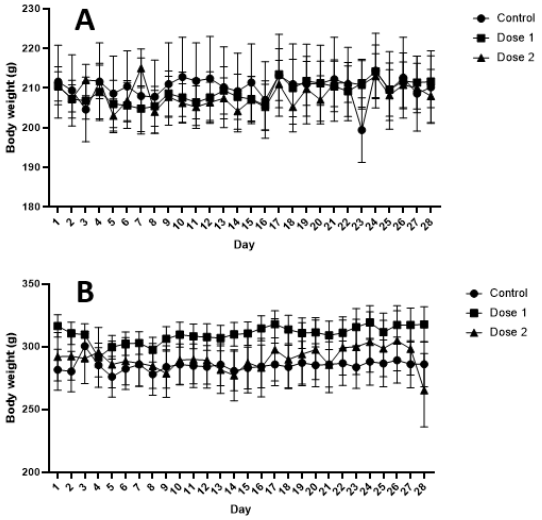

Figure 4. The bodyweight profile for each group of acute toxicity test. Male rats (A) and female rats (B). Data are means ± SEM (n=10) *P<0.05 compared to control

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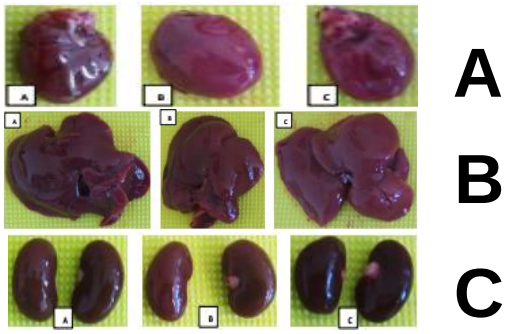

Figure 5. The visual appearance of the heart (A), liver (B), and kidney (C) organs for each group. (a) control group; (b) group I; and (c) group II

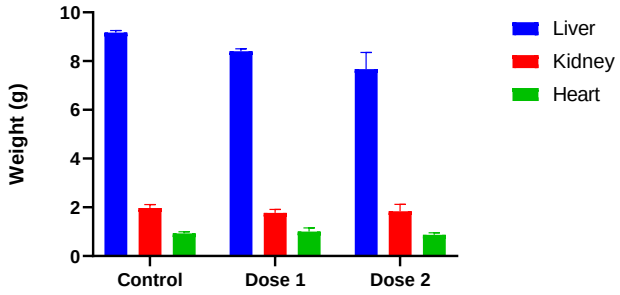

Figure 6. Each group's organs' weight profile of liver, heart, and kidney. Data are means ± SEM (n=10) *P<0.05 compared to control

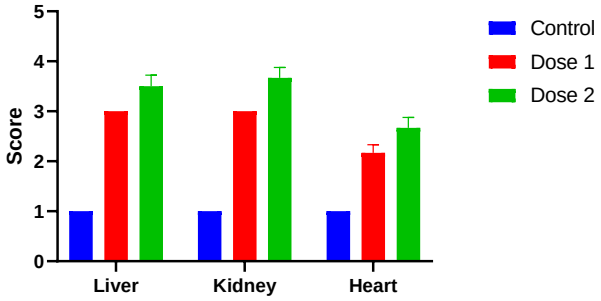

Figure 7. The organs' histopathological toxicity score profile of the rats. Data are means ± SEM (n=10) *P<0.05 compared to control

Organs histopathological toxicity score

It is known that administration of IHDM allergenic extract causes Histological changes in the liver, kidneys, and heart in the form of lesions on organs. Based on the graphic below, it is known that there was the difference in scoring scores in the liver, kidneys, and heart of experimental animals between groups. There is an increase in the score in test groups 1 and 2 compared to the control group. The greater the score obtained, the higher the score lesion or damage that occurs in the organ.

According to Ningsih et al. (2017), a change in body weight is one of the most accessible indicators visible. It is also an early indicator of the toxic effects of giving a sample of given test preparation. On the results of observations weighing in acute toxicity tests and subchronic toxicity during the trial period, not known to occur significant weight change between groups. The result found that animals' average body weight every day fluctuated, and generally no weight loss. It can be caused by feeding, where feeding will directly affect the weight of experimental animal bodies (Ningsih *et al.*, 2017).

Observation of liver, heart, and kidney organs was carried out in this study because the liver is an organ that acts as a site of metabolism drugs and toxic materials that enter the body and play a role in the system of blood flow to and from throughout the body through the

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system hepatic portal (Insani *et al.*, 2015). While the kidney has a volume high blood flow, carrying toxic cells through the tubules, and is an organ that produces urine, where urine is the major route of excretion of most toxicants (Makiyah & Tresnayanti, 2017). The heart is prone to abnormalities due to chemical compounds due to the number of mitochondria in the heart muscle relatively large numbers so that they are susceptible to cardiotoxicity. Another reason for choosing the three organs is because these three organs are involved in the metabolism of nutrients, drugs, and toxicants (Makiyah & Tresnayanti, 2017).

The liver is a portal circulation organ that transports compounds to be absorbed in the GIT. Metabolism toxic to the liver continuously results in toxicity liver, producing one or more reactive metabolites. Metabolites These reactive molecules bind macromolecular cells (such as proteins and lipids) irreversible so that it can cause loss of function of the macromolecule. One of the toxicity targets in the liver is the endoplasmic reticulum, which plays a role in protein synthesis in hepatocytes and is also the site of reactive metabolites of xenobiotics formed. It causes the endoplasmic reticulum to become susceptible to toxicant targets and produces injury by dilatation (Roberts *et al.*, 2000).

In the heart, mitochondria play an important role in susceptibility to cardiotoxicity. Cardiotoxicity is caused by oxidative stress, which causes the release of reactive oxygen species (ROS). This ROS release can stimulate lipid peroxidase and oxidative damage to mitochondria and myocyte cell membranes. Increased oxidative stress may also lead to the expression of transcription factors such as nuclear factor kappa B cell (NF-kB) and activation of the NLRP3 inflammasome, which results in increased release of pro-inflammatory cytokines myocardium as TNF- and IL-1β (Shaker *et al.*, 2018).

Several factors cause the kidneys to be susceptible to toxicity (Zhao et al., 2012). Kidneys are organs that play an essential role in maintaining the balance of body fluids and maintaining plasma volume and in, plasma volume and acid-base balance. Kidneys also detoxify organs and excrete toxic waste products of body metabolism (Ernawati, Witjahyo & Ismail, 2018). The toxic metabolism process in the kidney has the same way as solution metabolism serum, namely by passive glomerular filtration, passive tubular diffusion, and active tubular secretion. The kidneys require a large amount of ATP to maintain their transport function. ATP requirements high levels of this can cause the kidneys to be more susceptible to exposure to various toxins and hypoxic conditions (Zhao *et al.*, 2012; Ernawati *et al.*, 2018).

Further toxicity test studies, such as chronic toxicity tests, are conducted to determine the presence of toxic effects arising from the administration of long-term IHDM allergenic extract that has not been seen in this research. Then, the satellite group's evaluation shows the continuous subchronic effect of administering IHDM allergenic extract.

CONCLUSION

Administration of a single dose of IHDM allergenic extract does not cause toxic effects characterized by the absence of mortality in experimental animals during the observation period and not found signs of toxicity in experimental animals. Repeated doses of IHDM allergenic extract can result in toxic effects starting at a dose of 0.09 mg, characterized by death in experimental animals and the difference between macroscopic and microscopic conditions of organs in the test group with the control group. Administration of IHDM allergenic extract dose of 0.09 mg given for 28 days can cause histopathological changes in the liver, kidney, and heart of experimental animals marked with the difference in scoring scores in the higher test group than the control group.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.K., M.T.; Methodology, J.K., M.T., A.S.B.; Validation, J.K., M.T., A.S.B.; Formal Analysis, M.R., Z.A.Z.; Investigation, Y.A.P., W.F.S.; Resources, M.R., Z.A.Z.; Data Curation, J.K., M.T.; Writing - Original Draft, Y.A.P., W.F.S.; Writing - Review & Editing, J.K., M.T.; Supervision, J.K., M.R.; Project Administration, J.K.; Funding Acquisition, J.K., M.R.

CONFLICT OF INTEREST

The authors report no conflict of interest in this study.

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