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Sub-Acute Toxicity of Pigment Derived from *Penicillium resticulosum* in Mice

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Pigments derived from *Penicillium* have different toxicities depending on the pigment components. This study was intended to evaluate the sub-acute toxicity of oral exposure of Balb/c mice to *Penicillium resticulosum* pigment. A total of 50 healthy adult male and female mice were divided into 5 treatment groups and different doses of pigment (0, 125, 250, 500 and 1000 mg kg^{-1} body weight) were orally administered. Oral feeding of pigment with doses 125 to 1000 mg kg^{-1} body weight daily to adult mice did not cause mortality nor any clinical abnormalities. There were no significant differences in body, liver and kidney weights, nor liver and kidney functions of mice when pigment was given orally with intake doses of 125 to 1000 mg kg^{-1} body weight daily for 28 d in comparison to mice without pigment intake (control groups). There was a slight difference in liver histopathology of mice exposed to 500 and 1000 mg kg^{-1} body weight of pigment for 28 d in comparison to mice control groups, although there were no differences in kidney histopathology. Thus, we can conclude that the pigment of *P. resticulosum* can be cathegorized as low toxic pigment and well tolerated at dose below 500 mg kg^{-1} body weight daily for 28 d.

Key words: mice, *Penicillium*, pigment, toxicity

Pigmen yang berasal dari kapang *Penicillium* diketahui mempunyai toksisitas yang berbeda tergantung pada komponen yang dihasilkan. Penelitian ini bertujuan untuk mengevaluasi toksisitas sub-akut secara oral pigmen dari *Penicillium resticulosum* pada mencit Balb/c. Sebanyak 50 ekor mencit jantan dan betina dewasa sehat dibagi menjadi 5 kelompok perlakuan masing-masing 5, dan pada masing-masing kelompok diberi pigmen secara oral dengan dosis yang berbeda yaitu diberi 0, 125, 250, 500, dan 1000 mg kg^{-1} bobot badan per hari selama 28 hari. Mencit yang diberi pigmen 125-1000 mg kg^{-1} bobot badan tidak menunjukkan adanya kematian dan kelainan klinis. Fungsi hati dan ginjal mencit yang diberi asupan pigmen 125-1000 mg kg^{-1} bobot badan setiap hari selama 28 hari tidak berbeda signifikan dibandingkan dengan mencit yang tidak diberi asupan pigmen (control). Terdapat sedikit perubahan profil histologi hati mencit yang diberi asupan pigmen dengan dosis 500 dan 1000 mg kg^{-1} bobot badan setiap hari selama 28 hari dibandingkan profil histologi hati mencit yang tidak diberi asupan pigmen namun tidak terdapat perbedaan profil histology ginjal mencit pada semua dosis asupan pigmen dibandingkan dengan kontrol. Dapat disimpulkan bahwa pigmen dari *P. resticulosum* termasuk kategori toksik rendah dan aman dikonsumsi di bawah dosis 500 mg kg^{-1} bobot badan setiap hari selama 28 hari

Kata kunci : mencit, *Penicillium*, pigmen, toksisitas

The use of synthetic pigments as dyes has an important role in the food industry (Himri *et al*. 2011). However, since consumption of food containing certain synthetic pigment is known to cause health problems (Duran *et al*. 2002; Babitha *et al*. 2006), public attention towards the development of pigment from materials of natural origin has been increasing (Aberoumand 2011). As manufacturers demand rises for naturally-derived ingredients, particularly in food applications, naturally derived colorants appear set to overtake synthetic colorants in market value (Mapari *et al.* 2009). It is predicted that naturally derived pigment will have a bright future with a predicted annual growth rate of 5-10% while synthetic dye color are forecasted to grow at a lower rate of between 3 and 5% (Downham and Collins 2000).

There have been searches to find new sources of natural pigments from plants and animals to replace the synthetic pigment. Microorganisms have lately been receiving more attention as sources of natural pigment (Mapari *et al.* 2005). Production of pigments by fermentation has a number advantages; possibly easier extraction, higher yields, no lack of raw materials, no seasonal and agroclimate variations, and can be produced in a short period of time (Mortensen 2006; Aberounmand 2011; Poorniammal *et al*. 2011).

Microorganisms are known to produce various types of polyketide pigments, such as carotenoids, fenazine, acilphenol, pyrone, sclertiorine, and anthraquinone. Only carotenoid pigments and polyketide are known to be toxic (Poorniammal *et al*. 2011). Meanwhile, various species of fungi found in nature have reported but only a few have been explored

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for the purpose of production of food dyes (Mapari *et al*. 2006).

Production of pigment by the genus *Penicillium* is more efficient and profitable than the production of pigment by bacteria and yeasts. *Penicillium* can secrete enzymes and pigments out of the cell. The secreted pigment is relatively stable, thus easily purified. *Penicillium* can also grow on a variety of lignocellulosic materials. In previous studies, a *Penicillium* indigenous pigment producer was obtained from soil of Baluran National Park Indonesia and tentatively identified as *Penicillium resticulosum* based on morphological characteristics. However, the toxicity of pigment from this fungus is not yet known. This study aims to evaluate the sub-acute toxicity of pigment produced by *P. resticulosum* in mice.

MATERIALS AND METHODS

Fermentation and Pigment Production. *Penicillium* sp. was obtained from the laboratory of microbiology collection, Faculty of Mathematics and Natural Sciences, The Universitas of PGRI Adi Buana Surabaya Indonesia. Pigment production by *P. resticulosum* performed on liquid fermentation using a formulation media consisting of 5 mg KH_2PO_4, 0.2 g $MgSO_4$. H_2O, 3.0 g $NaNO_3$, 4.0 g of NH_4NO_3, 3.0 g yeast extract, and 30.0 g carboxymethylcellulose (CMC) in 1000 mL of hydrolysed corn cob medium with initial pH of 5.5. Medium was placed in Erlenmeyer flask, homogenized and sterilized in an autoclave at temperatures 121 °C for 15 min, then cooled to room temperature. All flasks were inoculated with 10 mL of the spore suspension containing 10^6 cells mL^{-1} conidia of *P. resticulosum* (estimated based on haemocytometer count). Fermentation was carried out in batch condition at 28-29 °C, 50% relative humidity with 60 rpm agitation for 12 days under dark condition. After incubated, whole of fermented matter was mixed with 200 mL distilled water and filtered through Whatmann No. 1. Filter followed by centrifugation at 3000 x g (Hettich EBA 8S Germany) for 15 min to separate pigment from spore and other material. One liter ethyl acetate was added to an equal volume of supernatant pigment and the mixture was adjusted to pH 3.0 with 2 N HCl with vigorous hand mixing and then let to stand for 15 min until two layers were formed. The ethyl acetate layer (upper layer) was obtained by pipette and evaporated using a rotary vacuum (Heidolph VV2011, Germany) at 50 °C. The pigment was collected and dried to constant weight at 50 °C for 48 h.

Experiments on Animals and Pigment Administration. A total of 50 healthy male and female Balb/c mice aged 3 months, weighing 30-35 g were obtained from PUSVETMA Surabaya and used for 28-days sub-acute toxicity evaluation. The mice were acclimatized for 14 days and assigned to five groups each consisting of five males and five females. The mice were housed in polypropylene cages (5 mice in one cage) on soft chip bedding, changed twice per week in a controlled room with a 12 hours light-dark cycle and temperature 26 ± 2 °C with relative humidity of 55%. They were kept with free access to water and dry commercial pellets feeding. Pigment *P. resticulosum* was fed by oral doses 0 (control), 125, 250, 500, and 1000 mg kg^{-1} body weight daily in NaCMC (sodium carboxymethylcellulose) daily for 28 d. Prior to dosing, animals were fasted overnight. Animals were observed thoroughly for onset of any immediate toxic signs and also during observation period of one week. General behaviors observed for the first 1 h, 24 h, and one week of test pigment administration were motor activity, tremors, convulsions, straub reaction, aggressiveness, pilo-erection, loss of lighting reflex, sedation, muscle relaxation, hypnosis, analgesia, ptosis, lacrimation, diarrhea and skin colour. At the end of the study (28 d), all animals were measured for the following variables; body, liver and kidney weight, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and liver and kidney histopathology.

Blood Sampling. Blood sampling was done by the cardiac puncture after the mice were anaesthetized by chloroform. Blood samples from each mouse were collected in tubes containing EDTA. Blood samples were stored for 1 hour and centrifuged in the cold at 2500 x g for 10 min. Serum was separated and placed in sterile plastic vials stored at -20 °C until use.

Biochemical Studies. Activity of serum Aspartate aminotransferase (AST**)** and Alanine aminotransferase (ALT) were determined by kinetic method (Teco Diagnostics). Blood urea nitrogen (BUN) was determined by Enzymatic Colorimetric method (Chronolab Cat. No. 101-0248). Alkaline Phosphatase (ALP) was determined by kinetic method (Diachem Ltd Cat. No. 48263). Lactate dehydrogenase (LDH) was determined by modification of Scandinavian Comitee on Enzymes (Chemhouse Cat. No. 065-0150).

Histophatological Studies. After 28 d pigment treatment, mice were anesthetized with chloroform and

sacrificed. Liver and kidneys were removed from abdominal cavity and weighed. Liver and renal samples of the control and treated mice were fixed in 10% phosphate buffered formalin for 24 h and then the samples were dehydrated and embedded in paraffin. Sections of 5 µm were done and stained with hematoxylin and eosin stains for histopathological studies under a bright field microscope with a magnification 400 times. A semi-quantitative analysis was done to assess the extent of the histopathological changes (Wang *et al.* 2000).

Statistical Analysis. The statistical significance of the differences between control and experimental groups was evaluated by Student's t-test using Statistical Package for the Social Sciences 13.0 (SPSS 13.0) software.

RESULTS

Mortality, Clinical Signs, Body and Organ Weights. There was no difference found in the mortality and clinical abnormalities in mice treated *P. resticulosum* pigment in comparison to control group (Table 1). However, we found one aggressive male mouse in the group that was given a dose 1000 mg kg^{-1} body weight *P. resticulosum* pigment daily. Absolute body, liver, and kidney weight in both male and female mice fed *P. resticulosum* pigment did not differ significantly (P>0.05) in comparison to control group mice. There were no significant (P>0.05) difference in the ratios of liver to body weight and kidney to body weight in comparison to control group mice (Table 2).

Biochemical Studies. There was no significant difference (P>0.05) in the level of AST, ALT, ALP, LDH and BUN in both male and female mice (Table 3).

Histopathological Liver and Kidney. There were no specific abnormalities of liver histopathology profile in the mice treated with *P. resticulosum* pigment (125 and 250 mg kg^{-1} body weight daily) for 28 d in comparison to that of the control group (Fig 1). Very mild sinusoidal congestion was found around the vascular central vein as a response to chloroform anesthesia. The lobular and sinusoids appeared normal and no congestion of sinusoids was seen at liver of mice treated with doses 0 (Fig 1A), 125 (Fig 1B), and 250 (Fig 1C) mg kg^{-1} body weight *P. resticulosum* pigment, but fat degeneration and necrosis in some lobus were found in the liver of mice treated with 500 (Fig 1D) and 1000 (Fig 1E) mg kg^{-1} body weight *P. resticulosum* pigment daily for 28 d.

Observations (Fig 2) of the pigment effects on kidney histology of mice showed that there was no found abnormal glomerulus, tubular necrosis, and atrophy in the kidneys of mice treated dose 0 (Fig 2A), 125 (Fig 2B), 250 (Fig 2C), 500 (Fig 2D), and 1000 (Fig 2E) mg kg^{-1} body weight *P. resticulosum* pigment for 28 d.

Table 1 Mortality and clinical signs of Balb Mice /c treated with oral administration of *P. resticulosum* pigment for one week

Clinical sign	*P. resticulosum* pigment dose (mg kg^{-1} body weight daily)									
	0 (control)		125		250		500		1000	
	M	F	M	F	M	F	M	F	M	F
Mortality	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Motor activity	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tremors	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Convulsions	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Straub reaction	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Aggressiveness	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5
Pilo-erection	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Loss of lighting reflex	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Sedation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Muscle relaxation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hypnosis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Analgesia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Ptosis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Lacrimation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Diarrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Skin color	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

M: male of five measurements; F: female of five measurements

Table 2 Effect of oral administration of *P. resticulosum* pigment on body, liver, and kidney weight of mice

	P. resticulosum pigment dose (mg kg^{-1} body weight daily)				
	0 (control)	125	250	500	1000
Absolute Body Weight (g)					
Prior treatment					N.D.
Male	29.18±2.36	31.64±3.63	30.98±0.97	30.42±2.36	27.86±3.63
Female	26.20±1.30	27.20±1.92	27.40±1.14	26.01±1.58	26.80±3.63
On day 28					
Male	29.38±2.32	31.78±3.63	31.10±0.83	30.66±2.31	28.46±3.22
Female	26.40±1.34	27.46±1.84	27.66±1.12	26.32±1.51	27.06±0.83
Liver					
Male	1.37±0.054	1.41±0.084	1.40±0.021	1.40±0.058	1.35±0.075
Female	1.30±0.019	1.33±0.054	1.32±0.045	1.29±0.022	1.28±0.027
Kidney					
Male	0.51±0.046	0.52±0.059	0.51±0.016	0.51±0.027	0.51±0.066
Female	0.44±0.005	0.47±0.014	0.44±0.010	0.44±0.014	044±0.007
Relative (% g $animal^{-1}$)					
Liver					
Male	4.72±0.22	4.49±0.25	4.53±0.12	4.61±0.18	4.89±0.40
Female	4.97±0.19	4.88±0.18	4.83±0.09	4.97±0.23	4.79±0.09
Kidney					
Male	1.74±0.04	1.64±0.02	1.65±0.09	1.66±0.06	1.71±0.02
Female	1.75±0.13	1.70±0.07	1.70±0.08	1.77±0.12	1.67±0.02

Values represent the mean ± SD of five measurements, $P<0.05$ indicates significant difference from controls.
N.D.: not detected

Table 3 Effect of *P. resticulosum* pigment administration on biochemical parameters of mice

Parameters	*P. resticulosum* pigment dose (mg kg^{-1} body weight daily)				
	0 (control)	125	250	500	1000
AST (UL^{-1})					
Male	76.02±0.75	76.04±1.11	76.70±1.27	77.01±1.27	77.84±0.72
Female	83.62±1.33	84.19±0.88	84.74±1.50	85.86±1.38	85.96±2.08
ALT (UL^{-1})					
Male	75.15±0.81	74.97±0.52	75.80±1.13	75.73±0.92	76.40±1.09
Female	74.63±1.31	75.18±0.97	75.52±1.68	75.54±0.76	76.35±118
ALP (UL^{-1})					
Male	38.30±1.80	38.81±2.35	40.72±1.31	40.16±0.95	40.42±0.69
Female	34.70±1.45	34.81±2.19	36.52±1.03	36.56±0.95	37.02±1.23
LDH (UL^{-1})					
Male	1288.47±67.62	1342.59±54.31	1348.74±30.23	1351.77±45.82	1367.54±28.80
Famale	1199.51±72.58	1249.64±55.64	1269.86±39.88	1280.03±36.71	1263.79±47.33
BUN (mg dL^{-1})					
Male	16.26±3.32	15.94±3.35	16.06±3.60	17.94±3.01	15.90±3.07
Female	16.90±2.38	18.28±1.12	16.68±2.40	18.10±1.26	18.14±1.55

Values represent the mean ± SD of five measurements, $P<0.05$ indicates significant difference from controls.

DISCUSSION

Evaluation of oral toxicity using 28 d toxicity tests generally has been conducted in sub-acute toxicity study which is the fundamental test to determine the level of product safety(Arts *et al*., 2004: Wang *et al*. 2007; Poorniammal *et al*. 2011), including the safety of food coloring pigments produced by microorganisms

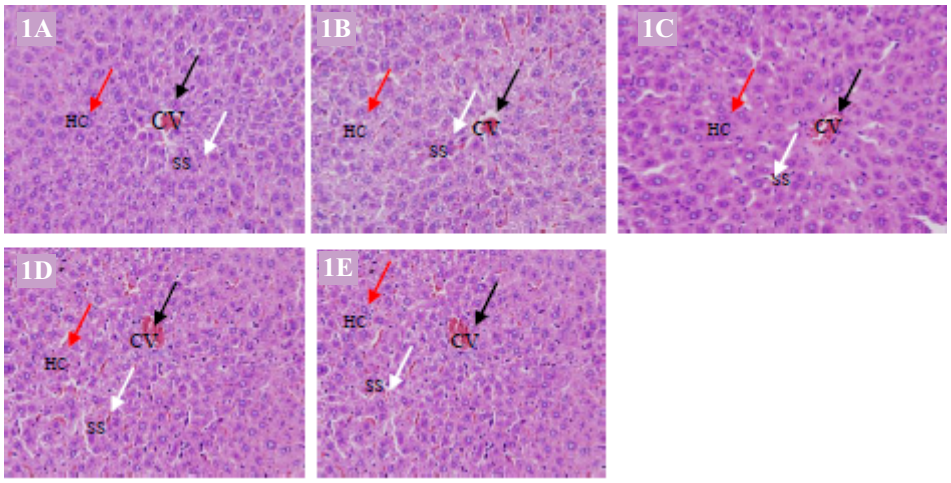

Fig 1 Liver section (magnification 400 x) of mice treated with orally administered various doses of *P. resticulosum* pigment: 0 (A), 125 (B), 250 (C), 500 (D), and 1000 (E) mg kg^{-1} body weight daily for 28 d. Vena centralis (CV) marked with black arrows, hepatocyte cell (HC) marked with red arrow, and sinusoids (SS) marked with white arrows.

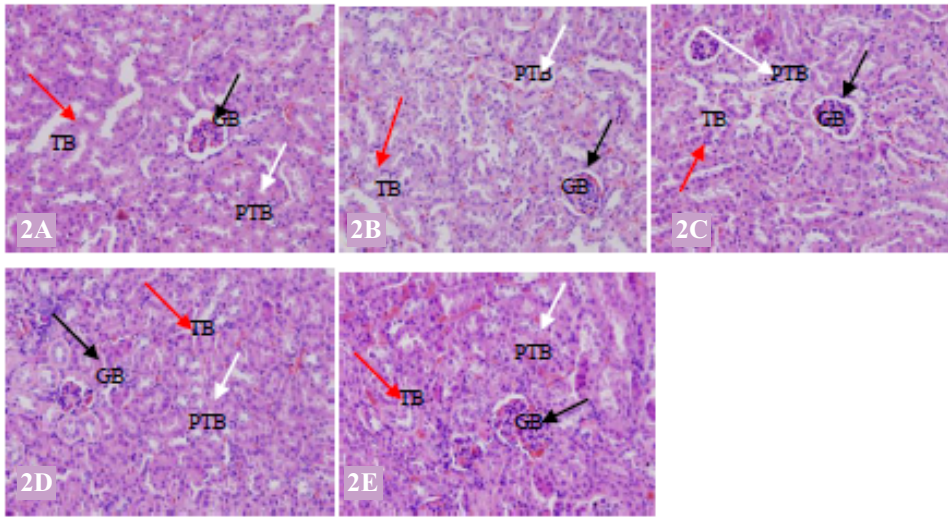

Fig 2 Kidney section (magnification 400 x) of mice treated with orally administered various doses *P. resticulosum* pigment: 0 (A), 125 (B), 250 (C), 500 (D) and 1000 (E) mg kg^{-1} body weight per day for 28 days. Glomeruli (GB) marked with black arrows, tubules (TB) marked red arrows, and proximal tubules (PTB) marked with white arrows.

(Kumari *et al.* 2009). In this study, during the administration period, no death occurred in animals from any group. There were no abnormal signs in the groups. Pigments from *Penicillium* have varied toxicity depending on the comonents of the pigment. Pigment omponent such as atrovenetin and norherqueinone from *P. antrovenetum* (Raistrick *et al*. 1958), antrovenetin and herquinones from *P. herquei* (Robinson *et al*. 1992), anthraquinone from *P. citrinum* (Duran *et al*. 2002), phoenicin from *P. antrosanguineum*, xanthoepocin from *P. brevicompactum*, anthraquinone derivatives arpink red from *P. oxalicum* (Mapari *et al*. 2005), mitosrubin, mitosrubinol and purpurogenone from *P. purpurogenum* (Mapari *et al*. 2006) have been known as non-toxic pigment. On the other hand, toxic pigment components include citrinine from *P. citrinum* (Duran *et al*. 2002), and acid-secalonic D from *P. oxalicum* (Mapari *et al*. 2005). Toxicity of pigment produced by *P. resticulosum* has not been reported by previous investigators. Shridhar *et al*. (2009) reported that the culture filtrate of *P. resticulosum* did not contain mycotoxins and 1.5 mL of culture filtrate of *P. resticulosum* only causes the death of mice after administration of the filtrate for 45 d.

Body and organ weights and ratio of organ with body weight are indicators of organ damages or abnormalities due to provision of treatment (Poorniammal *et al*. 2011). This study indicates that the administration of *P. resticulosum* pigment for 28 d produced no effect on the body weight gain and abnormalities in liver and kidney of mice. Shridhar *et al.* (2009) reported that although the administration of

1.5 mL of culture filtrate of *P. resticulosum* gave no effect on the body weight of mice, the body weight decreased gradually until the provision of 45 d.

Activities of AST, ALT, ALP, and LDH are indicators of liver damage. Our study demonstrated that the daily intake of *P. resticulosum* pigment for 28 day exhibited no significant effect on AST, ALT, ALP, and LDH when compared to the both male and female control mouse. The culture filtrate of *P. resticulosum* with a dose of 1.5 mL per day for 45 d can increase the concentration of AST and ALT enzymes, indicating liver damage (Shridhar *et al*. 2009). Fumaryl-dl-alanine (Fumaromono-dl-alanine) is a metabolic product of *P. resticulosum* that might have caused altered permeability and/ cell necrosis, leaked of the enzymes into the bloodstream (Birkinshaw *et al* 1942).

Consumption of synthetic or natural food colorant after 30 days of treatment increased serum creatinine and urea in rats (Helal *et al*. 2000; Himri *et al*. 2011). Blood urea nitrogen is an indicator of kidney damage as a result of enzymatic hydrolysis of urea by urease to ammonia (Zafar *et al.* 2010). Our study showed upon the administration *P. resticulosum* pigment in mice for 28 days there were no differences in BUN levels in both male and female mice compared to mice control groups.

Loss of the structure and abnormal function of cells are the results fat metabolism disorder in cells, inducing degeneration. Cells undergoing fatty degeneration was characterized by the accumulation of metabolic products such as molecules of fat, protein and glycogen in abnormal amounts. Fatty degeneration of the cells indicates the presence of biochemical disturbances caused by abnormal metabolism. Toxic chemicals that are microscopically visible as grains of fat accumulated in the liver lobes, especially the perilobular tissue. The present study revealed that mouse consuming high dose of *P. resticulosum* pigment (500 and 1000 mg kg^{-1} body weight) daily for 28 days exhibited slight existence of fatty degeneration and necrosis in some lobus.

Kidney proximal tubule is the most vulnerable to damage from toxic substances. In the proximal tubule occur the process of absorption and secretion of various substances. If there is absorption of toxic materials in the tubular epithelium, it would interfere with the metabolism and absorption. In addition, levels of cytochrome P-450 in the proximal tubule increased to detoxify toxic substances. In the present study, microscopic observations of kidneys histopathologic preparations found no abnormalities in glomeruli, tubules, proximal tubules and capillaries between the tubules.

In conclusion, the 28 d sub-acute toxicity evaluation indicates that pigments produced by *P. resticulosum* can be categorized as low toxicity pigments. Provision of *P. resticulosum* pigment up to a dose of 500 mg kg^{-1} body weight daily for 28 days had no effect on body weight, organ weights, and activities of AST, ALT, ALP, LDH enzymes and BUN. However, mouse taking *P. resticulosum* pigment above 500 mg kg^{-1} body weight daily showed fatty degeneration and mild necrosis of liver cells indicating that the consumption of pigment from *P. resticulosum* was still safe up to doses below 500 mg kg^{-1} body weight daily for 28 d.

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