The Preventive Effect of Ethanolic Extract of Rome Beauty Apple Peel (*Malus sylvestrys* Mill) towards Protease Activity and Jejunal Histopathology of Rat (*Rattus norvegicus*) Exposed to Lead Acetate

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**Abstract.** The exposure of lead to body can be through inhalation, digestion and adsorption. Lead intoxication can lead to jejuna damages and increase protease activity due to reactive oxygen species (ROS) production. Apple peel extract contains flavonoids that can scavenge ROS. This research aims to investigate the effect of ethanolic extract of Rome Beauty apple peel to protease activity and jejuna histological feature of rat exposed to lead acetate. This research used 20 rats, that divided into 5 treatment groups, which were negative control group (healthy rat), positive control group (exposed to lead acetate 10 mg/rat/day for 14 days), and preventive therapy group (administrated the apple peels extract with doses of 28 mg/200 g BW, 56 mg/200g BW, 112mg /200g BW for 21 days and also lead acetate with 10mg/rat/day for 14 days on day 15th until 28th). Jejunal histological features was stained using HE stain which then observed using a microscope. Protease activity was measured by using spectrophotometric method. The data of protease activity was statistically analyzed using ANOVA, followed by Tukey test (α = 5%). The results showed that the administration of ethanolic extract of Rome Beauty apple peels at best dose of 112 mg/200 g BW can reduce protease activity and improve jejuna histopathology. The conclusion of this study was that apple peels extract of Rome Beauty can be used to prevent the increase of protease activity and the damage of rat jejuna exposed to lead acetate.

1. Introduction

Lead is a heavy metal used for manufacturing batteries, paints, greases and oil for car engine, farm machinery and pipes. Lead is important toxic metal because of the danger to both human and animal health. Animals exposed to lead poisoning mainly via oral routes, the pastures and water contaminated by lead which is accidentally consumed and can cause intoxication. Lead poisoning seldom occur by airways due to the prolonged inhalation of lead fumes and absorption of lead through the skin [1]. Lead toxicity in adult cow is at dose of 400-600mg/kg (single dose), however, it defends of the lead compounds. Chronic toxicity occurs on cow, whether fed grass containing Pb 390 mg/kg daily. Cow and poultry kept near landfill are the most at risk of lead poisoning because they are inquisitive and commonly taste the new finds. In addition, in cow meat was found Pb levels of 0.05-1.05ppm [2], while in chicken meat was 0.56-4.83ppm [3].
When commercial animals contaminated by lead are consumed by human, that may cause health problem. Lead enter the body via digestive tract that will be absorbed by intestine. Symptom of lead poisoning in gastrointestinal system include anorexia, abdominal pain, vomiting and constipation [4]. The accumulation of absorbed toxic material can disrupt the adsorption system in small intestine especially jejunum. The presence of lead can form free radical and increase ROS level in body without the equal increase of endogenous antioxidant leading to the occurrence of oxidative stress, that consequently, can damage cell and organs. Cell damage can trigger macrophage activity and inflammatory cell to produce pro-inflammatory cytokines such as TNF-α, then it activates neutrophils to produce protease [5].

Current therapy of lead poisoning can be conducted by using chelating agents which can bind lead and facilitate its release. The chelating agent, meso-2,3–dimercaptosuccinic acid (DMSA) is used orally, while ethylene diamine tetra acetate (EDTA)is used intravenously and intramuscularly. Those chelating agents can reduce blood lead levels. However, there is no information of the changes of morphological organ [6]. In order to overcome lead poisoning in animals, the effort may use natural product such as apple peel instead of apple flesh. Apple peel contains flavonoid, ascorbic acid, phenolic compound, glutathione and L-cysteine [7]. It has been previously reported that ethanolic extract of apple peel at dose of 28mg/kg BW can reduce MDA levels and fat degeneration in rat hepatocytes induced by streptozotocin [8].

In this work using rat model, the rats were orally induced by lead acetate and received preventive therapy with ethanolic extract of Rome beauty apple peel. We reported the activity of jejuna protease activity and jejuna histopathology changes exposed to different dose of ethanolic extract of Rome beauty apple.

2. Materials and Methods

Eight to ten weeks old, male Wistar rats weighing about 180-200g were allowed to acclimatize for 7 days prior to initiation of the study. The animals were housed in completely controlled environment (room temperature 25 ± 2 °C and 12 h light and dark cycle) with free access to water and food. The Research ethics committee (animal care and use committee) of Brawijaya University has approved the study protocol (No. 1087 –KEP-UB).

2.1 Chemicals and Instrumentations

The materials used in this study were fruit peel of Rome beauty Apple which obtained from local market and determined by PT. Materia Medica, Batu City, East Java. Other materials used were protease reagents (PBS-Tween solution, PMSF, Trichloro acetic acid (TCA), buffer phosphate pH 7.0, absolute ethanol, bovine serum albumin (BSA), L-tyrosine, paraffin, 10%paraformaldehyde (PFA), xylol. paraffin block, PBS –azida solution pH 7.4, hematoxylin and eosin.

2.2 Experimental Animals and Design

Twenty male Wistar rats were divided into five groups. Each group consist of 4 rats. Group 1 was the control group (healthy rats). Group II was the positive control (Pb poisoning). Group III, IV and V rats were designated as experimental group and received ethanolic fruit peel extract of apple orally in a dose of 28, 56 and 112/200gBW/day, respectively by gavage for 21 days. Group II, III, IV and V received lead acetate at a dose of 10mg/200gBW/day for 14 days on day 15th until 28th. At the end of experiments, all rats were sacrificed, and jejunum organ were isolated for determining protease activity and observing histopathology feature.

2.3 Isolation of protease and measurement of protease activity

The isolated jejunum organ was weighted for 0.5 g and cut into small pieces by using surgical scissors. Then it was added with 1mL of PBS-tween solution: PMSF (9:1), crushed in cold mortar and transferred to sterile micro tube that used addition of 2 mL of PBS-tween solution: PMSF (9:1). The mixture was put into a vortex vibrator for 10 min, sonicated for 10 min, and centrifuged for 10 minute 6000 rpm.
Supernatant was separated and precipitated by absolute ethanol at volume ratio of 1:1 and allowed for 24 hours. The mixture was centrifugated at 10000 rpm for 15 minutes. Finally, the precipitate was protease which was dried until free ethanol and dissolved in 0.02M Tris-HCl solution pH 6.5. The protease activity was measured using spectrophotometer based to Walter Method with equation:

$$\text{protease activity} = \frac{\text{tyrosin}}{M_r \text{ tyrosin}} \times \frac{V}{P \times q} \times f_p$$  \hspace{1cm}(1)$$

where $V$ = total volume, $P$= volume protease, $q$= time reaction (minutes) and $f_p$ =dissolution factor.

2.4 Jejuna histopathological Observation

Histopathology observation of rat jejunum were carried out by using a light microscope Olympus BX51 with magnifications of 400x. The observations were especially in mucosal layer with vile, the presence of hyperplasia goblet cells, epithelial erosion, infiltration of macrophage and neutrophils.

2.5 Statistical Analysis

Data of protease activity were expressed as the mean ± standard deviation of the mean and comparison between different treatments was carried out using one-way ANOVA through software SPSS 22.0 followed by Tukey test. Significance was accepted at $p<0.05$.

3. Results and Discussion

Protease has been considered to act primarily as degradative enzyme in extracellular space, however their biological actions in tissues and cells suggest important roles as a part of body hormonal communication system during inflammation and immune response. The lead acetate induction to rats can damage jejunum and increase the jejunum protease activity. The protease activity can be measured by spectrophotometry. The effect of preventive therapy of ethanolic extract of apple peel to jejuna protease activity of rats induced by lead acetate were displayed in Table 1.

**Table 1.** Profile of jejuna protease activity in control rats, Pb poisoning rats, and Pb poisoning rats with preventive therapy with ethanolic extract of Rome beauty apple peel.

| Group             | Protease activity (μmol/mL min) |
|-------------------|-------------------------------- |
| 1. Negative control | 4.08 ± 0.08$^a$               |
| II. Pb poisoning   | 5.99 ± 0.37$^c$               |
| III. Therapy 28mg/kgbw | 4.84 ± 0.38$^b$           |
| IV. Therapy 56 mg/kgbw | 4.45 ± 0.17$^{ab}$        |
| V. Therapy 112 mg/kgbw | 4.25 ± 0.16$^a$             |

*different letters (a-c) show significant statistical different effects in each group ($p<0.05$).

As shown at Table 1, the negative control rats show jejuna protease activity of 4.08±0.08 μmol/mL min, which is significantly different compared to positive control rats. Protease is normally an intracellular enzyme which has a function as signal transduction and cell regulation to activate polypeptide hormone, development factor and body defense. In development factor, protease has a function as collagen assembly from procollagen, cell proliferation, proteolytic control and plays a role in apoptosis. Moreover, protease can be used as an indicator of severity of inflammation [9].

The increase of protease activity of rat jejenum induced by lead acetate was caused by lead inhibition of δ ALAD activity that can increase the accumulation of ALA. The increase of ALA resulted on the formation of free radicals, peroxide radical, superoxide radicals and hydroxyl radicals [10]. Free radicals cause cell damage, thus triggering the activity of macrophages in tissue to secrete pro inflammatory cytokines, namely tumor necrosis factor (TNF-α). The TNF- α subsequently activate neutrophils to produce protease.
Therapy with ethanolic extract of apple peel may reduce the elevated jejuna protease activity. Protease activity decreased with the increasing doses of ethanolic extract of apple peel. Statistical test results showed that there were significantly differences ($p<0.05$) between protease activity in rat jejunum induced with lead acetate and rat jejunum with therapy. It suggests that flavonoid in ethanolic extract enable to act as an antioxidant, especially as hydroxyl radical scavenger and suppress the increase of protease activity. The role of flavonoid as antioxidants directly is by donating hydrogen electron to free radicals and increasing the expression of endogenous antioxidant genes [11]. In addition, ethanolic extract of apple peel contains vitamin C that can act as antioxidant. The suppression of oxidative stress can decline the activation of NF-κB, consequently, TNF-α cannot be produced. The decline of TNF-α will decrease the activity of neutrophils as first immunity defense that will suppress the protease activity [12].

The damage of rat jejuna exposed to lead acetate can be observed by the changes of histopathological jejuna instead of jejuna protease activity. Free radicals are the result of normal cell metabolism; however, lead poisoning may interfere the balance between free radicals and endogenous antioxidants leads to situation of oxidative stress. The oxidative stress can cause damage to jejunum tissue and jejunum dysfunction. The histopathology image of jejunum was observed to determine both the level of damage and organ repair. Comparison of histopathological jejunum image from normal rats, lead poisoning rat and therapeutic rats were displayed in Figure 1. In negative control rats, there are neat arrangement of columnar epithelial cell and goblet cells in tunica mucosa and the infiltration of inflammatory cells do not exist. Normally, jejunum consists of four layers, tunica mucosa, tunica submucosa, tunica muscularis and tunica serous [5].

The positive control rats (K+) showed a different image of histopathological jejunum mucosa compare to negative control as shown by the damage of villous structure, hyperplasia goblet cell, epithelial cell erosion and rupture of villi and infiltration of macrophages and neutrophils. Similar finding by Sharma and Barber, that lead induction can change histopathology in small intestinal mucosa such as damage to villous structure, epithelial cell erosion and infiltration of inflammatory cells. Villi are the most important part of the small intestine with function of absorption in digestive tract. Intact villi will facilitate the transportation of nutrient to the body. Damage to villous structure occurred due to erosion of epithelial cell that will lose a portion of mucosa [13]. Goblet cells spread between absorptive cells that functions of producing mucus to protect mucosal epithelium and facilitate the movement of food ingested which is not absorbed. The synthesized mucus consists of a mixture of water, glycoprotein, glycolipids, electrolytes, enzymes, salts and glandular secretions. Lead induction will cause intestinal response to toxic compounds by multiplying goblet cells to produce a lot of mucus as jejunum defense. Therefore, some hyperplasia goblet cell existed in positive control rats

Improvement in histopathologic image of rat jejuna induced by lead acetate can be seen in therapeutic groups. Improvement is evident from the decline of erosion of epithelial cells, hyperplasia goblet cells and infiltration of inflammatory cell (macrophages and neutrophils). In therapeutic groups treated with a dose of 112mg/kg bw show an improvement in the histopathological features of rat jejuna which is almost the same as negative control group where the villous structure is neatly arranged, hyperplasia goblet cell and inflammatory cell are not found. It suggests that flavonoid and vitamin C in ethanolic extract of apple peel can neutralize free radicals and prevent the occurrence of protein, DNA and lipid peroxidation, thus consequently, can prevent the damage of rat jejuna.
Figure 1. Histopathology features of normal rat jejuna (I), Pb poisoning rats (II), Pb poisoning rats and treated with ethanolic extract of apple peel (III, IV, V) at magnifications of 400x. goblet cells (↑), infiltration of inflammatory cells (↑), villous erosion (↑), villous rupture (↑).

4. Conclusion
It can be concluded that ethanolic extract of apple peel has bioactive compounds as preventive agents to lead poisoning. A dose of 112 mg/200g BW/day is the effective dose to prevent damage of rat jejuna induced by lead acetate based on the decrease of protease activity and improvement of jejuna histopathological feature in the form of reduced goblet cell hyperplasia, infiltration of inflammatory cells and epithelial cell damage in villi compared to positive group. The results indicate that ethanolic extract of apple peel is potential to be used to prevent lead poisoning. Further study is necessary to package the extract. Instead of giving solution of apple peel extract by force feeding, it needs for giving therapy in simple way such as giving the extract in different form, like capsule, tablet, or feed mixture. Therefore, the stability of extract in the package should be also examined.

References
[1] Traverso S D, Loretii A P, Donini M A and Driemeier D 2004 Arq. Bras. Med. Vet. Zootec. 56 418-421.
[2] Janardani N MK, Berata I K, and Kardena I M 2018 Indonesia Medicus Veterinus. 7 42-50 In Indonesian.
[3] Priyono O 2013 Study of Pb levels and distributions in chicken meat, liver, and Kidney, Faculty of Veterinary medicine, Bogor Agricultural University. In Indonesian.
[4] Lubis B, Rosdiana N, Nafianti S, Rasyianti O and Panjaitan F M 2013 Relationship of lead poisoning and Iron Deficiency in Children. CDK 200, 40(1) 17-21 In Indonesian.
[5] Huang C, Šali A, Stevens RL, Regulation and function of mast cell proteases in inflammation. J Clin Immunol. 1998; 18 169–183.
[6] Mason LH, Harp JP and Han D Y 2014 Pb neurotoxicity: Neuropsychological effects of lead toxicity. BioMed Research International 2014: 1-8.
[7] Lata, B., and Tomala K. 2007 Apple peel as a contributor to whole fruit quantity of potentially healthful bioactive compound. cultivar and year implication. J. Agric. Foods Chem. 55(26) 10795-10802.
[8] Andriani S R D 2016 The effect of ethanolic peel extract of apple Manalagi (Mallus Sylvestris Mill) towards MDA levels and hepatic histopathology feature of diabetic Type 1 rat. Faculty of Veterinary Medicine, University of Brawijaya. In Indonesian.
[9] Chapman H A, Riese R J and Shi G P 1997 Emerging roles for cysteine protease in human biology. Annu. Rev. Physiol. 59 63 – 88.
[10] Gurer-Orhan H, SabirH U and OzgunezH 2004 Correlation between clinical indicators of lead poisoning and oxidative stress parameters in control and Lead exposed workers toxicology. 195 147-154.
[11] Purwaningsih I, Sapriani R dan Indrawati R 2018 Antioxidant Activity of methanolic extract of Daun Kesum (Polygonum minus Huds.) by DPPH method. Jurnal Laboratorium Khatulistiwa. 2(2) 161-165. In Indonesian.
[12] Chattopadhyay I, Bandyopadhyay U, Biswas K, Maity P and Banerjee R K 2006 Indomethacin inactivates gastric peroxides to induce reactive-oxygen-mediated gastric mucosal injury and curcumin protects it by preventing peroxidase inactivation and scavenging reactive oxygen. Free Radical Biology Med. 40 1397-1408
[13] Sharma R and Barber I2014 Lead toxicity and postnatal development of gastrointestinal tract. Universal Journal of Environmental Resarch and Technology. 4(3) 121-133.