Chronic Hepatotoxicity Test of Cassava Leaves Extract (*Manihot utilissima* Pohl.) on Wistar Rat (*Rattus norvegicus* L.)

Nugrahaningsih W.H[1]

[1]Jurusan Biologi, Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Negeri Semarang, Indonesia

Abstract

Cassava leaves have a huge potential to be developed as phytopharmaca for orthostatic hypotension. Cassava leaves contain minerals as the agent of anti-hypotension. The safety of long-term consumption of cassava leaves remains unidentified scientifically therefore it is necessary to test its' in-vivo chronic toxicity. The test focused on the liver (hepatotoxicity) because the liver is the first organ exposed to xenobiotics that enter the body. This research is an experimental research with time-series design. This study used 36 Wistar rats divided into four treatment groups with various doses of cassava leaves extract given at 0, 80, 400, and 2000 mg/kg for 90 days. The hepatotoxicity parameters are the value of SGOT, SGPT, as well as body weight measurement that observed every 30 days. Clinical toxicity symptoms were observed daily during the treatment. At the end of the study, liver organ was taken to observe the morphology and to measure the relative weight of liver. This research procedure included the extraction of cassava leaves using maceration method, treatment by giving the cassava leaves extract orally, and measure the value of SGOT and SGPT using spectrophotometric methods. The research data was analyzed using linear regression and one way ANOVA. The cassava leaves extract that given to the Wistar Rat for 90 days gave a significant effect on the value of SGOT after 60 days. The value of SGPT, weight, as well as relative weight of the liver haven't significantly affected by the cassava leaves extract. Clinical toxic symptoms were experienced by group with dose of 400 mg/kg in the form of nose and mouth bleeding on day 14 and stopped on day 32, and it experienced by group with dose of 2000 mg / kg in the form of severe bleeding in the nose and mouth on day 12 and continues to cause infection. Mortality cases were experienced by group with dose of 2000 mg/kg on day 27 and 41

INTRODUCTION

Cassava (Manihot utilissima Pohl.) is an agricultural plant that widely used as a source of carbohydrates for people. Particular part of this plant—cassava leaves—are commonly consumed by some African and Asian people. Cassava leaves contain 5-7% crude protein, 1-2% crude fat, and 2% minerals in dry weight. The amino acids in cassava leaves are equivalent to the egg whites, with the exception of methionine, lysine and isoleucine. The protein content in cassava leaves are much higher than the daily protein intake recommended by Food and Agriculture Organization (FAO) and it is higher than soy protein, spinach leaves, oats, and rice (Ferraro et al., 2016; Latif & Muller, 2015).

Generally, cassava leaves are consumed as vegetables especially by Javanese people of Indonesia. Moreover, cassava leaves also used as herbs with various purposes such as to overcome orthostatic hypotension (Nugrahaningsih et al., 2017). Cassava leaves contain minerals such as sodium (Na), potassium (K), and iron (Fe) which can perform as anti-hypotension agents (Nugrahaningsih et al., 2017; Dickson et al., 2012).

Orthostatic hypotension which also known as postural hypotension is a common cardiovascular disorder that with or without signs of underlying neurodegenerative disease (Ricci et al., 2015). Orthostatic hypotension occurs when systolic blood pressure drops into ≥20 mmHg or diastolic blood pressure increases ≥10 mmHg within three minutes when the body changes position to stand with a slope of at least 60° (Freeman et al., 2011). The long-term effects of the orthostatic hypotension—if it doesn't treat immediately—can result to heart failure. The study that conducted by Jones et al. (2012) has proven that orthostatic hypotension can increase the risk factors for diabetes mellitus, hypertension, and coronary heart disease

which are the main causes of heart failure. In order to overcome the orthostatic hypotension, it requires an appropriate and an effective treatment.

Unfortunately, cassava leaves also contain a hydrogen cyanide compounds (HCN) which at certain doses can be very toxic for livings (Ferraro et al., 2016). This toxicity is caused due to its ability to inhibit the activity of metalloenzymes, especially cytochrome c oxidase which is the final enzyme of the electron transport chain respiration (Gleadow & Moller, 2014). Due to this toxic compounds, it is required an effort to develop cassava leaves into phytopharmaca orthostatic hypotension with BPOM standards needs. It also supported by preclinical data such as toxicity data in order to know the safety information and possible side effects (*PerKBPOM Number 13 of 2014*). Therefore, this study aims to test the chronic toxicity of cassava leaves extract in Wistar Rat (*Rattus norvegicus L.*).

The study of toxicity focuses on the liver organ (hepatotoxicity) for three main reasons including (1) the liver is the first organ exposed by xenobiotics that enter the body through the digestive tract; (2) in the process of detoxification by enzymes in the liver metabolites are often produced which can actually damage the liver; (3) xenobiotics that accumulated in the bile will be released in the intestine and transported back to the liver thereby increasing the concentration of xenobiotics in liver hapatocyte cells (Hodgson & Levy, 2004; Wallace & Meyer, 2010). The hepatotoxicity parameters in this study included blood biochemical levels called Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT) levels, body weight growth, relative liver weight, and clinical toxic symptoms.

RESEARCH METHOD

Experimental Animal

All 36 of healthy Wistar Rats were divided into four groups which are one control group and three treatment groups. Each group consists of nine Wistar Rats. The control group (K) was a group that was not treated with cassava leaves extract—only given 10 mL of distilled water. The treatment groups P1, P2, and P3 were given cassava leaves extract with doses of 80, 400 and 2000 mg/kg, respectively. Rats were kept in cages with the size of 30x45 cm^2, and acclimatized for 14 days before being treated.

Extraction of Cassava Leaves

Cassava leaves were initially prepared—including cleaning, drying and refining processes—and extraction process by then. Cassava leaves are cleaned by rinsing water then it was dried in an oven at temperature of 50°C for 24 hours. Cassava leaves then be blended into dry powder. The dry powder then added with distilled water in a ratio of 1: 5. This suspension is then stirred and heated at the temperature of 70°C for an hour using a magnetic stirrer then incubated for five days. After the incubation process was complete, the supernatant is separated to be evaporated in an oven to obtain the dry powder/thick liquid of cassava leaves extract.

Research Procedure

Cassava leaves extract are then diluted according to the particular dose then be given to Wistar Rats orally for 90 days using a gastric-sonde. Clinical toxic symptoms such as the changes in behavior, secretion, respiratory rate, eye color, stool consistency, bleeding, swelling, and mortality were observed during the treatment. Measurement the value of SGOT, SGPT and body weight was observed every 30 days of treatment, while the relative weight of the liver was measured at the end of the treatment.

The measurement of SGOT and SGPT values were initiated by taking blood from the capillary hematocrit through rat retro-orbital sinus and save in 1.5 mL of micro-tube. Blood was centrifuged at 4000 rpm in 10 minutes. Blood serum was taken with a 30 µL micropipette then mixed with SGOT reagent and 300 µL SGPT in the micro-tube. This mixture was then homogenized with vortex and transferred to the cuvette for its absorption at 340 nm wavelength against blank by spectrophotometry. As blanks, that was used distilled water with a volume of 330 µL.

Data Analysis

The hepatotoxic effect of cassava leaves extract which was given chronically to Wistar Rats in the control group and treatment group was known through linear regression statistical tests, while to find out the differences in each treatment group was using one way ANOVA test.

RESULT

Hepatotoxicity testing of cassava leaves extract on Wistar Rats was carried out for 90 days. The hepatotoxicity parameters that observed in the study were SGOT and SGPT values, weight, relative liver weight, and clinical toxic symptoms. During the treatment period, there were various considerations including, the group with dose of 2000 mg/kg had to be terminated at day 45 of treatment due to experiencing symptoms of severe cyanogenic glucoside poisoning which is continuous bleeding in the mouth and nose area caused by infection. Symptoms of poisoning were also seen in the group with dose of 400 mg/kg, but the bleeding stopped after entering the second month of the treatment.

The results of linear regression of statistical analysis of the SGOT data in Table 1 were obtained by $p > 0.05$ at month 1 and 2, while in the 3rd month it has the value of $p < 0.05$. The value of R^2 obtained in of the 3rd month treatment shows the effect of cassava leaves extract by 21.1%. Comparative statistical analysis with one way ANOVA technique was obtained $p > 0.05$ at month 1 and 2, while in the third month the value of $p < 0.05$. Further LSD test obtained p value < 0.05 in group K with P2, and $p > 0.05$ in group K with P1 and P1 with P2.

Table 1. The SGOT value of Wistar Rat that received cassava leaves extract in oral for 90 days.

Dose	Month- (U/L)*		
	1	2	3
K (0 mg/kg BW)	120,18±29,74	135,64±24,84	157,72±28,23[a]
P1 (80 mg/kg BW)	146,97±39,41	168±56,94	183,54±63,84[ab]
P2 (400 mg/kg BW)	118,46±33,30	135,78±22,08	222,42±59,53[b]
P3 (2000 mg/kg BW)	142,41±42,81	x**	x**

*value of mean±SD obtained from 3 replications
**Wistar Rat was terminated at day 45

Statistical calculation of the effect of linear regression on SGPT Rat data for every month was obtained $p > 0.05$. The R^2 value per month shows the effect of cassava leaves extract on rat SGPT levels of only 8.6%, 2%, and 0.4%. Comparative statistical analysis with one way ANOVA method obtained the value of $p < 0.05$ only in the 1st month. after the LSD, it was found that group K with P1, group K with P2, and group K with P3 produced a value of $p < 0.05$. While the P1 group with P2, P1 with P3, and P2 with P3 resulted in a value of $p > 0.05$.

Table 2. The SGPT value of Wistar Rat that received cassava leaves extract in oral for 90 days.

Dose	Month- (U/L)*		
	1	2	3
K (0 mg/kg BB)	47,58±9,65[a]	66,47±13,03	69,04±12,49
P1 (80 mg/kg BB)	71,34±14,30[b]	79,60±16,40	87,23±23,85
P2 (400 mg/kg BB)	67,58±27,19[b]	75,94±19,54	77,38±12,50
P3 (2000 mg/kg BB)	74,93±27,80[b]	x**	x**

* value of mean±SD obtained from 3 replications
** Wistar Rat was terminated at day 45

Statistical analysis of the effect of using linear regression data on body weight every month resulted the value of $p > 0.05$. While the comparison analysis using one way ANOVA was resulted the value of $p<0.05$ after the 3rd month of treatment. Further LSD test obtained the value of $p<0.05$ only between groups K with P1, while between groups K with P2 and group P1 with P2 obtained the value of $p>0.05$.

Table 3. The body weight of Wistar Rat that received cassava leaves extract in oral for 90 days.

Dose	Month- (g)*		
	1	2	3
K (0 mg/kg BB)	172,22±37,85	195,22±36,61	209,44±44,10[a]
P1 (80 mg/kg BB)	148,11±34,27	158,11±31,40	158,67±23,45[b]
P2 (400 mg/kg BB)	157,67±37,69	168,78±29,79	186,33±34,82[ab]
P3 (2000 mg/kg BB)	147,11±27,37	x**	x**

* value of mean±SD obtained from 3 replications
** Wistar Rat was terminated at day 45

Statistical analysis of the influence using linear regression obtained the value of $p>0.05$. R^2 value shows the effect of various doses of cassava leaves extract on the relative liver weight of only 0.6%. The difference between each group is known by the one way ANOVA analysis that resulted the value of $p>0.05$.

Tabel 4. Relative weight and liver morphology of Wistar Rat that received cassava leaves extract in oral for 90 days.

Group	Relative Wight of Liver (g)*	Morphology of Liver
K	0.0371±0.0102	- Liver's color and surface are normal - 2 rats have hydatid cyst on liver**
P1	0.0500±0.0124	- Liver's color and surface are normal - 4 rats have hydatid cyst on liver **
P2	0.0468±0.0096	- Liver's color and surface are normal - 9 rats have hydatid cyst on liver **
P3	0.0459±0.0142	- Liver's color and surface are normal - 8 rats have hydatid cyst on liver ***

* value of mean±SD obtained from 3 replications
** hydatid cyst was founded at day 90 of treatment
*** hydatid cyst was founded at day 45 of treatment

DISCUSSION

Cassava leaves extract with various doses gave effect the value of SGOT Rats after 60 days of treatment, with a percentage of influence of 21.1%. The average of SGOT value showed a significant difference in the 3rd month of treatment. Significant differences were seen starting at the dose between the control groups at a dose of 400 mg/kg.

Various doses of cassava leaves extract didn't give a significant effect to the SGPT value of Wistar Rat's blood. The effect of cassava leaves extract on the levels of SGPT Rats per month experienced a decrease that was only 8.6%, 2%, and 0.4%. This enhancement indicates the body's adaptation of Wistar Rat to the cassava leaves extract orally thus in the 3rd month the value of the influence reached less than one percent. The real difference is that the average of SGPT level is only found in the 1st month, while the second and third months are similar. At the first month, there are significant differences between the control group with a dose of 80 mg/kg, the control group with a dose of 400 mg/kg and the control group with a dose of 2000 mg/kg.

Gad (2007) stated that SGOT and SGPT values of normal Rats were in the range of 45.7-80.8 U/L and 1.5-30.2 U/L, but in this study the SGOT value of all groups were far above the normal range. (Table 1 and 2). This can be caused by several factors, such as the presence of a virus or worm infection, fat degeneration that can be seen through histopathological examination, and traumatic muscle injury due to fights during the treatment period (Lu, 1995; Bayard, 2006; Kee, 2008). In this case, the high values of SGOT and SGPT results of the study are thought to be strong due to parasitic worm infections which then form a cyst in the liver.

Cassava leaves extract has no effect on Rat's body weight. The average weight of Rats showed a significant difference in the 3rd month of treatment, between the control groups with a dose of 80 mg/kg. Whereas between the control group with a dose of 400 mg/kg and a group with the dose of 80 mg/kg and 400 mg / kg were no difference between those two groups.

According to Ferraro et al. (2016) cassava leaves contain of rich calories, fat, carbohydrates and fiber. However, in this study the cassava leaves chronically had no effect on Rat's weight. This is because cassava leaves are given in the form of extracts, so that the calorie, fat, carbohydrate, and fiber content of cassava leaves are significantly reduced or even lost when the extraction process.

Liver observation in this study was done macroscopically by looking at the relative weight, color, and surface of the liver. The relative weight of the liver was calculated according to the ratio of liver weight to rat body weight. The relative weight of the liver is a parameter that is assessed in determining the toxicity of the test substance. This is because some types of liver were damage such as inflammation that can affect the relative weight of the liver (Relle et al., 2005).

The morphology of Rat's liver was observed by looking at the color, surface, and abnormalities that exist on the liver. According to Kumar et al. (2013) normal liver morphology has a flat and smooth surface and has brownish red, while the abnormal liver morphology has surfaces such as connective tissue, spots, cysts, as well as discoloration. Based on the results of the study, all rats' hearts had a brownish red color and a smooth and flat surface, but several rats were found to have abnormal conditions in the form of cysts.

The further identification revealed that the cyst was a hydatid cyst caused by Echinococcus spp parasitic worm infection. Infective stages were caused by adult worms or larvae (metasestoda) of Cestoda species. Rats and other rodents are intermediate hosts. Hydatid cysts are formed after the egg that enters the body breaks and releases the oncosphere which penetrates to the intestinal wall and carried by the circulatory system to the liver. The cyst will continue to enlarge and produce protoskoleks and child cysts (daughter cyst) which then fill the cyst space (Sandy, 2014).

This parasitic worm infection is difficult to identify because it does not provide a specific clinical symptom (asymptomatic) and tolerant of surrounding tissues/organs. The most effective way to find Echinococcus spp infection is by histopathological observation and macroanatomy organ observation. In this study Rats may have been infected with Echinococcus spp. before the treatment held, so it was not related to the provision of cassava leaves extract. Echinococcus infection in this study is categorized as an uncontrolled variable that cannot be avoided.

Although the surface of the liver is hydatid cyst, but based on the statistical analysis it does not affect the relative weight of the liver. This is because the cyst is only an empty space containing a little liquid. Further research is needed regarding the specific species of Echinococcus spp. and efforts to avoid the possibility of transmission to humans, because it concerns the health of researchers.

Rat clinical toxic symptoms were occurred during the treatment of cassava leaves extract. The results of the examination of clinical toxic symptoms are summarized in table 5. From the observations, found conditions that showed bleeding, there were even two cases of mortalities. Both cases of mortalities occurred in group Rats with dose of 2000 mg/kg on days 14 and 41. In this case three characteristics were found, including (1) symptoms of bleeding seen from day 14 of treatment with severe bleeding in the mouth and nose area; (2) before being found dead the Rat experienced decreased activity, weakness, and decreased appetite; (3) when found dead found dry blood spots in the mouth and nose area of rats.

Rat mortalities are estimated to occur due to the cassava leaves cyanide poisoning which causes tachycardia and or internal haemorrhage in the area of the Rat's respiratory system. Cyanide poisoning from cassava leaves can cause arrhythmic symptoms in the form of tachycardia where the heart rhythm is faster than normal conditions and tracheal which results to the bleeding in the mouth and nose area. Other symptoms that may arise include increased respiration rate and depth, no response to stimuli and spasmodic muscle movements, darkening of muscle tissue, and blockage and lung bleeding (Constable et al., 2017; Uhegbu et al., 2012).

Oral and nasal bleeding was experienced by seven rats in the dose of 400 mg/kg group and all rats in the group with dose of 2000 mg/kg. Both groups began bleeding when they entered the 14th day of treatment, but on the 32nd day of bleeding in seven rats in the 400 mg/kg dose group appeared to stop. Whereas in the group with dose of 2000 mg/kg occurred the bleeding and even worse with the marking of infection in three rats in the mouth area as well as the weakening physical condition every day. Considering this and considering the ethical research clearness aspect, on the 46th day all rats in the group with dose of 2000 mg/kg were terminated so as not to torture the animal.

Not all groups of Wistar Rat were given cassava leaves extract experienced bleeding or mortality conditions, bleeding experienced by the group dose of 400 mg/kg and 2000 mg/kg, while mortality only occurred in the group of 2000 mg/kg. So it is estimated that cassava leaves extract will cause toxicity effects at a dose of 2000 mg/kg and the safe limit for consumption of cassava leaf extract is at a dose of 400 mg/kg.

The dose of cassava leaves extract given to Rat 200 g if converted to 70 kg human dose using the calculation of Brunton et al. (2008), the dosage of cassava leaves extract for humans are 0, 4.48, 22.4, and 112 g. According to the results of this study showed that the safe threshold for consumption of cassava leaf extract in adult humans is 22.4 g per day, because at the threshold of doses above 22.4 g can cause the toxic symptoms of cyanogenic glucoside poisoning clinics and abnormalities of fat metabolism in the liver.

CONCLUSION

The extract of cassava leaves at low and middle doses have no chronically toxic effects on rats, while it has toxic effect on rats at very high dose. The symptoms of toxicity appear in the form of continuous bleeding in the area of the nose and mouth as well as mortality.

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