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Acute and Subchronic Toxicities of Indonesian Mistletoes *Dendrophthoe pentandra* L. (Miq.) Ethanol Extract

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ABSTRACT

Traditionally mistletoes *Dendrophthoe pentandra* (L.) Miq known in Indonesia is to cure cough, hypertension, diabetes, cancer, ulcers, smallpox, diuretic, skin infection and after child-birth. The objective of this study was to evaluate the toxic effects at short and long term the *Dendrophthoe pentandra* ethanol extract in mice. In the acute test, the limit test dose of 40 g/kg of aqueous and hydroalcoholic extracts were administered orally to mice and then observed individually 2 h post-dosing and at least once daily for 14 days. Sub-chronic toxicity was evaluated after a daily oral administration of 420 mg/kg in a suspension of 2 % PGA for 90 days to Wistar rats. Animals were sacrificed and their organs were examined. The results showed LD_{50} values for acute toxicity at a dose of 17.78 and 12.59 g/kg which was comparable to a dose of 12.45 g/kg and 8.81 g/kg in rats. From the subchronic, the values of the parameters of hemoglobin, hematocrit, leukocytes, and erythrocytes index were still within the range of the reference. From histopathological examination value, the results revealed some abnormalities. Our results suggest the ethanol extract of *Dendrophthoe pentandra* have LD_{50} values which have practically not toxic but is not recommended to be used for a long periode.

INTRODUCTION

Indonesia is known as a country with a great amount of plant biodiversity that is widely used as traditional medicines. One example of this traditional medicine is *Dendrophthoe pentandra* L. Miq. Traditionally,the mistletoe *Dendrophthoe pentandra* (L.) Miq is known in Indonesia to cure cough, hypertension, diabetes, cancer, ulcers, smallpox, , skin infection and for its therapeutic value after child-birth or as a diuretic. Mistletoe *Dendrophthoe pentandra* L. Miq. is sold in stores as a herbal tea and may be used to treat breast cancer,although it is actually a parasite of the mango tree (Hutapea, 1993; Dalimartha, 1999; Depkes RI. 2000). In Sulawesi, this plant is traditionally used as ananti-cancer agent (Ishizu *et al*., 2002; Zainuddin and Sul'ain, 2015). Katrin *etal.* (2005) her dissertation, claimed to isolate quercetin from mistletoe leaves *Dendrophthoe pentandra* (L.) Miq. Similarly, Artanti *et al.* (2006) isolated a flavonol glycoside, quercitrin (quercetin-3-O-rhamnoside) from star fruit (*Averrhoa carambola*) and mistletoe (*Dendrophthoe pentandra* (L.) Miq.). Quercetin fromplant extracts can be determined as the total alkaloid based on method of Chang *et al.* (2002).*In vivo* quercetin has been reported to have anti-inflammatory activity at a dose of 10 mg/kg body weight (Gusdinar *et al*., 2011). Quercetin content in *Vitex trifolia* L. was reported to have anti-inflammatory effect (Mustarichie *et al*., 2016).Quercetin isolated from leaf mistletoe has also been shown to inhibit L1210 leukemia cells (Katrin *et al*., 2011). Artanti *et al.* (2012) reported *in vitro* toxicity, antioxidant and antidiabetic activities of methanol and water extracts of *D. pentandra.* Fitrilia *et al.* (2015) reported regarding their phytochemical screening and reported on the antioxidant activity of clove mistletoe *D. pentandra*.

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We have previously reported ourstudy onthe interaction of quercetin and casticin with H4r, anti-inflammatory receptor, as supporting data for the anti-inflammatory herbal medicine Mustarichie *et al.*, 2012, as well as on the total flavonoid content and anti-inflammatory properties of Indonesian mistletoe *Dendrophthoe pentandra* L. (Miq.) ethanol extract (Mustarichie *et al.*, 2015). Wicaksono and Permana (2013) also stated *Dendrophthoe pentandra* potential as an anti-cancer agent colon.

In general, toxicity test methods can be divided into two groups (Loomis, 1978; Gossel and Bricker, 199; Lu, 1995). Acute toxicity tests are designed to determine the median lethal dose (LD_{50}) of a toxicant. LD_{50} is defined as the single dose of a substance that can kill 50% of test animals as described by Gossel and Bricker (1990). These types of tests quantify the acute toxicity of drug compounds, provide a therapeutic index (TI) or a safety limit for the particular drug compound, and provide an estimation of the therapeutic dose and initial dose of a drug study. The TIis an expression of the relationship between the therapeutic dose and the lethal dose and is calculated as the ratio of the LD_{50} to the ED_{50}, i.e. TI= LD_{50} / ED_{50}). Subchronic toxicity evaluations are used to determine the safety of use of a compound for a longer period of time (PPOM, 1991).

In this research, the acute and subchronic toxicity tests of *Dendrophthoe pentandra* ethanol extract in mice were carried out in order to obtain an overview of its safety margin for when it will be developed into a standardized herbal medicine.

MATERIALS AND METHODS

Experimental animals

Male and female mice (*Mus musculus*) weighing between 20–30 g and 2–3 months old, were obtained from the School of Pharmacy, Institute of Technology Bandung, Indonesia. Before use, the mice were acclimated for one week and their body weight was measured every day. Mice were said to be healthy if the weight increased or decreased by no morethan10% of the previous weight with normal activity during the acclimation period. Ethical approval for this study was issued by the Kementerian Riset, Teknologi dan Pendidikan Tinggi, Fakultas Kedokteran Universitas Padjadjaran, Komisi Etik Penelitian Kesehatan No. 108/UN6.C.1.3.2/KEPK/PN/2015.

Extraction method

In order to ensure the sample (mistletoe *Dendrophthoe pentandra*) used was from the same source throughout the experiment, the fresh sample was collected in sufficient quantities (~10 kg) at a time (Ahmad *et al*., 2009). The sample was collected from Subang area, West Java. First, the plant was washed thoroughly with running tap water, followed by rinsing with distilled water and then each part was cut into small pieces and powdered. They were sun dried (~30 ℃) at open area with active ventilation until they attained constant weight (around three weeks). The Soxhlet apparatus was used for extraction process in which the extractor thimble was fitted in between a round bottom flask at the bottom and a bulb condenser at the top. Inside the thimble holder, 200 g dried *Dendrophthoe pentandra* powder was wrapped within a packing. Extraction using 1.8 L ethanol 75 % was carried out till obtain clear droplets. This procedure was guided by Farmakope Herbal Indonesia (Departemen Kesehatan RI, 2015) and modified Gatbonton (2013) and Marnoto (2012) methods. The obtained extract was concentrated by using a rotary evaporator and freeze dryer.

Test preparation

The test preparation was made with a dose variation. Viscous extract was weighed according to the dose that had been determined and then prepared as asuspension using 2%PGA, respectively preparations made as many as 10 mL. Each of the test preparation was put into labeled vials.

Acute and subchronic toxicity methods

Acute and subchronic toxicity testing methods were based on modified Ouedraogo *et al*. method (2013). The OECD Guideline for Testing of Chemicals: 420 Acute Oral Toxicity-Fixed Dose Procedure (OECD, 2001) and The OECD Guideline for Testing of Chemicals: 408 Subchronic Oral Toxicity (OECD, 1998) as guidance for acute and subchronic tests, respectively. Toxicity studies of Pingale *et al*., (2011), Mridula *et al.* (2011) and Rais (2014) were also used as references to this study. Acute toxicity tests were conducted within 14 days, whereas subchronic toxicity tests were carried out for a period of 121 days. The subchronic toxicity tests used male and female white Wistar mice. Animals were grouped randomly into five groups of five mice each. The group given the test materials with different doses of a test group and a group of satellites, one group was given a liquid carrier as a negative group. Each test dose was administered orally for three months. The negative control group was given only the liquid vehicle. On day 91 of the experiment, the hematology and blood chemistry exams were conducted. Animals were sacrificed and their organs were examined and a histopathologic index was applied. The aim of this test was to determine the safety level for long-term use of the extract.

Data were analyzed statistically using analysis of variance design perfectly random. Observational data, such as the onset of toxic symptoms, were analyzed using the Friedman two-way analysis of variance for the acute toxicity test, whereas the subchronic toxicity data was analyzed using Student's t-test with a confidence level of 95% for the urine and blood parameters, blood biochemistry and organ indices. The parameters of body weight, gastric mucosal ulcer index and organ histopathology were conducted descriptively by direct observation of the presence or absence of change.

RESULTS &DISCUSSION

Extraction

A viscous extract with a concentration of 13.28% was obtained by soxhletation of mistletoe *Dendrophthoe pentandra*

using 95% ethanol. The resulting extract was dark green in color, and had a distinctive smell and bitter taste. The water content of the ethanolic *Dendrophthoe pentandra*extract was 3.89%. This value indicated that the ethanolic *Dendrophthoe pentandra* extract was of fairly good quality because the water content did not exceed the allowed moisture content standard of ≤ 4.1% (BPOM RI, 2004). That is, the extract could be stored for a long period of time without significant contamination by microbes or fungi.

Acute Toxicity Testing

The acute toxicity was evaluated through observation of mortality in mice over 14 days, observations of the weight of the mice for 14 days, and an observation ofthe mouse behavior for the first 24 hours after receiving the ethanolic extract of *Dendrophthoe pentandra*. The mortality data for the male and female mice is reported in Tables 1 and 2, respectively.

Table 1: Male mice mortality (%).

Mice	Cumulative Mortality (%)						
	2 h	4 h	24 h	48 h	72 h	7 d	14 d
Control	0	0	0	0	0	0	0
Dose I (5 g/kg BW)	0	0	10	10	10	10	10
Dose II (10 g/kg BW)	0	0	20	20	20	20	20
Dose III (20 g/kg BW)	20	40	40	40	40	40	40
Dose IV (40 g/kg BW)	40	50	70	70	70	70	70
Dose V (80 g/kg BW)	90	90	90	90	90	90	90

Table 2: Female mice mortality (%).

Mice	CumulativeMortality (%)						
	2 h	4 h	24 h	48 h	72 h	7 d	14 d
Control	0	0	0	0	0	0	0
Dose I (5 g/kg BW)	0	0	0	0	0	10	10
Dose II (10 g/kg BW	0	0	40	40	40	40	40
Dose III (20 g/kg BW)	10	10	40	50	50	50	50
Dose IV (40 g/kg BW)	50	80	80	80	80	80	80
Dose V (80 g/kg BW)	90	90	90	90	90	90	90

To calculate the value of LD_{50}, results of toxicity test data was drawn to a straight line between log dose vs probit percentage value. Probit percentage determined using probit table (Finney and Stevens, 1948) by changing the value of % of animal deaths to the percentage in the table probit. Having obtained a linear line equation, then the $LD_{50}$was calculated by changing the value of y to 5, the results obtained made his anti-Log (Kobayashi and and Pillai, 2013; Hayes and Dipasquale, 2001) as shown in Figure 1.

Curve of blue line shows the cumulative mortality (%) of male mice over the 14 day period, compared with that of the females (red line). Based on the Figure 1, it was found male and female mice had LD_{50} of 17.78 and 12.59 g/kg BW, respectively. By extrapolation, a dose of 17.78 g/kg in mice was comparable to a dose of 12.45 g/kg and 12.59 g/kg was comparable to 8.81 g/kg in rats. According to the criteria of Hodge and Sterner (Table 3), the results of toxicology LD_{50} value has meaning and it could be concluded that the ethanol extract pentandra *Dendrophthoe pentandra* in the range of doses was practically not toxic as LD_{50} is the dose range of 5–15 g/kg BW rat. These results were consistent with the results obtained by Katrin *et al.* (2005a), who found that up until a dose of 2000 mg/kg body weight of animal died and was no significantly toxic effects.

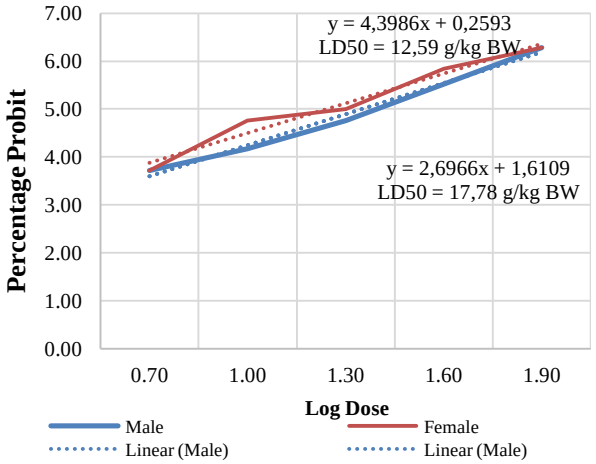

Fig. 1: Log Probit Analysis of *Dendrophthoe pentandra*.

LD_{50} of groups of males and females show different values. This is consistent with the theory put forward by Lazarovici and Haya (2002) which states that between males and females there are differences in sensitivity to a toxicant. The differences are influenced directly by the endocrine glands, therefore it can be said the gender differences affect the value of LD_{50}.

Table 3: A substance toxicity Assessment.

Toxicity levels	General terms	LD_{50}in rats (orally)
1	Unusually toxic	≤1 mg/kg
2	Very toxic	1–50 mg/kg
3	Quite toxic	50–500 mg/kg
4	Slightly toxic	0,5–5 g/kg
5	Practically non-toxic	5–15 g/kg
6	Relatively safe	>15 gkg

(Derelanko, 2008).

Observations on Body Weight

Daily body weight was observed for 14 days in both the male and female mice. This was conducted to determine if the extract had any effect on weight for the two week period following administration. These data were analyzed using analysis of variance (ANOVA). The results ofthe statistical testing demonstrated that there was no significant difference in body weight of the male and female mice as a result of the administration of different doses of the extract at a significance level of 0.05.

Subchronic toxicity testing

White Wistar rats were divided into three groups of five animals: a control group, a test group, and the group of satellites. Each group received their test preparation orally, once a day for 90 days. The control group was given the vehicle, a suspension of 2% PGA, and the satellite test group was given the ethanolic extract of *Dendrophthoe pentandra* at a dose of 420mg/kg in a suspension of 2% PGA.

Changes in Body Weight

No significant differences were observed in body weight following the administration of the *Dendrophthoe pentandra* ethanol extract test preparation at a dose of 420 mg/kg as compared with the satellite animals and the controls, that received vehicle only (Tables 4 and 5).

Blood Biochemical Examination Results

The blood biochemical tests are a very important aspect of the toxicology tests, and they are essential to understand the mechanisms of the disease process, specifically regarding the enzyme levels in the liver and kidneys.

A. Liver Function Test

The results of the liver function tests in mice are presented below (Table 6). SGOT and SGPT values were statistically different between the groups (0.018, $p < 0.05$). Data showed that the SGOT values were greater in the test group than in the control group, but the value was still approaching the reference value. The results of SGPT levels in the test group were smaller than the control group, but the overall value of SGPT was much larger than the reference value, indicating liver damage.

B. Kindey Function Test

The results of the kidney function test in mice are shown in Table7 below.

Animal Blood Test Parameters

The results of the blood panel are shown in Table 8. A blood panel, consisting of the parameters hemoglobin, hematocrit, leukocytes, erythocytes, thombocytes, and erythrocytes index (MCV, MCH, and MCHC) was carried out. Statistical analysis revealed no difference between groups for hemoglobin, hematocrit, leukocytes, and index erythocytes (MCV, MCH, and MCHC) because the value sig (0.018 <0.05). The values of the parameters of hemoglobin, hematocrit, leukocytes, and index erythrocytes were still within the range of the reference value, whereas the erythrocyte and platelet values for all groups, especially the test group and satellite group were well above the reference values. This was expected because the entry of foreign substances triggers the body's response with regard to bleeding and tissue damage.

Table 4: Rat body weight.

Group	H7	H14	H21	H28	H35	H42	H49	H56	H63	H70	H77	H84	H91
Test	236.39	241.14	238.57	243.29	243.32	243.89	246.04	244.71	246.61	248.25	250.00	244.07	210.11
Control	243.54	244.00	246.64	250.68	250.04	248.89	251.68	251.54	252.14	252.00	248.46	243.36	209.32

Notes: H = Testing times (days)

Table 5: Change inanimal weight

Group	H7	H14	H21	H28	H35	H42	H49	H56	H63	H70	H77	H84
Test	4.75	-2.57	4.71	0.04	0.57	2.14	-1.32	1.89	1.64	1.75	-5.93	-33.96
Control	0.46	2.64	4.04	-0.64	-1.14	2.79	-0.14	0.61	-0.14	-3.54	-5.11	-34.04
Satellite	4.11	4.14	5.96	-0.14	1.71	3.43	2.89	5.61	0.54	4.39	-1.50	-34.93

Notes: H = Testing times (days)

Table 6: Liver Enzymes: SGOT and SGPT Levels

Group	SGOT (IU/L)	Value Reference	Value SIG	SGPT (IU/L)	Value Reference	Value SIG
Test	148	141	0.018	57	12.6	0.018
Control	114	141	0.018	61	12.6	0.018
Satellite	134	141	0.018	66	12.6	0.018

Table 7: Creatinine Levels.

Group	Creatinine (mg/dl)	Reference Value	*p*-value
Test	0.25	0.2 ~ 0.8 mg/dL	0.018
Control	0.27	0.2 ~ 0.8 mg/dL	0.018
Satellite	0.28	0.2 ~ 0.8 mg/dL	0.018

Creatinine levels were significantly different among all groups ($p< 0.05$).However, creatinine values of all groups are still within the range of the reference value.

Table 8: Animal Blood Test Results

Blood Parameter	Group				
	Test	Control	Satellite	Reference value	SIG value
Hemoglobin (g/dL)	14.2	13.2	14.3	11.5~16.1	0.018
Hematocrit (%)	45	40	45	36~52	0.018
Leucocytes (/mm^3)	3,300	3,000	3,100		0.018
Erythocytes (mil/uL)	7.83	6.91	7.86		0.018
Thombocytes (mm^3)	1,112,000	870,000	1,251,000	150,000~450,000	0.018
MCV (fL)	57.5	57.9	57.5	48~70	0.018
MCH (pg)	18.1	19.1	18.2	17~21	0.018
MCHC (%)	31.6	33	31.6	35~43	0.018

Histopathological Examination Results

The histopathological examination results are shown in Table 9.

Table 9: Resultsof histopathological examination

Organ	Histological analysis
Liver	U: necrosisof hepatocytes (++), vacuolization (++), hydropic degeneration (+++), inflammation (++), bleeding (++) K: necrosis of hepatocytes (+), vacuolization (+), hydropic degeneration (+), inflammation (+) S: necrosis of hepatocytes (+), vacuolization (++), hydropic degeneration (++), inflammation (+), bleeding (+)
Kidney	U: Acute Tubular Necrosis (NTA) (+++), vacuolization and degeneration of the proximal convoluted tubule and distal convoluted tubules (+++), bleeding (++) K: vacuolization and degeneration of the proximal convoluted tubule and distal convoluted tubules (+), bleeding (+) S: Acute Tubular Necrosis (NTA) (++), vacuolization and degeneration of the proximal convoluted tubule and distal convoluted tubules (++), bleeding (+)
Heart	U: Bleeding (++), inflammation (+) K: Bleeding (+) S: Bleeding (+), inflammation (+)
Tumors	U: Lymphoma (+++) S: still visible remnants of connective tissue and inflammation

Notes: + = Slightly damaged
++=Moderate damage level
+++=Heavy damage level
U=Group test
K=Control group
S=Satellite group

The histopathological examination of test animals included an examination of the heart, liver, kidney, and tumors. In the most severe case, kidney damage found in the test groups, which manifested as acute tubular necrosis, vacuolization and degeneration of the proximal convoluted tubule and distal convoluted tubules, as well as massive bleeding. In the group of satellites, A cute Tubular Necrosis, vacuolization and degeneration of proximal and distal convoluted tubules lighter than thetest group. However, more severe than in the control group in which the control group was very mild abnormalities in the kidneys.

Histopathological examination of the liver showed hepatocytenecrosis, vacuolization, hydropic degeneration, inflammation, and severe bleeding. In the satellite group, the observed hepatocytenecrosis, vacuolization, hydropic degeneration, inflammation, and bleeding was not as severe as in the group test, but it was worse than in the control group. In the heart, bleeding and severe inflammation were present in the test group as compared with the control group and satellite group. From the observation Satelliteand Test group found any swelling suspected tumor. In the test group shown Lymphomas evere, and in the group of satellites was able to seethe rest of the connective tissue and inflammation. These tumors could be associated with a given dose of the extract, because they the control group, did not happen the same as in the other two groups were given the extract.

CONCLUSION

Acute toxicology testing revealed that the ethanolic extract of the herb *Dendrophthoe pentandra* had a LD_{50} of 17.78 and 12.59 g/kg which was comparable to a dose of 12.45 and 8.81 g/kg in rats. Based on the toxicity classification of Hodge and Sterner, it can be concluded that the ethanol extract of the herb *Dendrophthoe pentandra* can be categorized as practically non-toxic, but long term use is not recommended.

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