Acute Toxicity Test of Ethanolic Extract of Dayak Onion Leaves (*Eleutherine americana* Merr.) Toward Wistar Female Rats Using OECD 425 Method

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ABSTRACT: Pre-clinically, the potential of *Eleutherine americana* Merr. as antioxidant has been studied, but it's safety level of its safety has not been widely known. Safety level of ethanolic extract of *E. americana* Merr leaves (EEEaL) can be detected by acute toxicity test using OECD 425. The aim of this study was to investigate the acute toxicity of EEEaL as the guideline of its safe dose for therapy. This test was performed through OECD 425 (Up and Down Procedure) method with two doses (2000 and 5000 mg/kgbw) of EEEaL administration orally which observed for two weeks toward Wistar rats. The results of the test dose showed no toxic symptoms and they did not cause death in the test animals. Single dosage up to 5000 mg/kgbw also did not show any symptoms of toxicity, and did not cause weight loss until the 14th day of test. The LD_{50} value of EEEaL is more than 5000 mg/kgbw, suggesting that the plants is practically non toxic according to Loomis classification. Phytochemical screening showed that EEEaL contains compounds such as alkaloids, flavonoids, triterpenoids, steroids, and saponins.

Key words: *Eleutherine americana*, leaves, acute toxicity, OECD 425.

INTRODUCTION

Indonesia is world's third the most active smoker after China and India while the smoker number increases annually not only in Indonesia, but also all over the world.[1] Long term smoking affects closly related to prevalence of diseases such as chronic obstructive pulmonary disease (COPD), lungs cancer, atherosclerosis which caused adverse effect.[2] Each cigarette comprises a number of dangerous chemicals such as nicotine, carbon monoxide, tar, etc. One time smoking produces 10^{14-16} oxidant molecule within the smoke such as superoxide, hydrogen peroxide, hydroxyl, and peroxyl free radical.[3] Oxidant molecules can lead to oxidative stress characterized by increasing amounts of oxidant radicals, the total of leukocytes and blood neutrophils.[4] Free radical molecule from the cigarette smoke catalysts the peroxidation of double unsaturated fatty acid of cell membrane which strengthened oxidative stress level during smoking activity.[5,6] The longer somebody smokes the higher MDA level on their bodies.[7]

Dayak onion leaves plant or Mekah onion leaves also has antioxidant activity potential. According to the research of Andriyani (2014), it is known that Dayak onion extract has potential as an antioxidant against cigarette smoke exposure by decreasing MDA level. Another study also suggested that ethanolic extract of Dayak onion leaves may decrease the degree of lung damage caused by the exposure to cigarette smoke.[8] The acute toxicity test is a safety test in a short time to detect any toxic effects of a given test substance in a single or recurrent dose within 24 hours.[9] This test aims to investigate the safety of ethanolic extract of Dayak onion leaves administration orally toward test animals by observing several test parameters such as LD_{50}, body weight change, organ index, and behavior. Acute toxicity test results will give an insight of the acute

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reaction of living things when administered certain medicinal substance.[10]

MATERIAL AND METHODS

Sample collection. Dayak onion leaves were collected from Kubu Raya regency and then identified at the Laboratory of Biology in Faculty of Mathematics and Sciences UNTAN. The result showed that the official name of this species is *Eleutherine americana* Merr. with local name Bawang Dayak.

Extraction. The method of extraction that was conducted was maceration. One kg dried leaves of dayak onion was macerated with 1.5 litres of ethanol (70%) for 72 hours (3 days in a row). After every 24 hours, ethanol was replaced with the new ethanol solvent and macerate was stirred regularly. The next step of this macerate was to filter and evaporate the solvent using rotary evaporator.

Extraction standard. Specific parameters used in this research include organoleptic assay, soluble compound content, water soluble content, total flavonoid determination.

Non-specific parameters used in this research are moisture content, ash content parameter, bacterial total and mold total assay, Lead (Pb) limit determination.

Acute toxicity test using OECD 425 method. Acute toxicity test was performed using OECD 425 according to *Acute Oral Toxicity-Acute Toxic Class Method*.[9] The experimental animal in this research is female Wistar rats at the age of 6-8 weeks, with minimal body weight about 150 grams. LD_{50} value was obtained through counting the death and alive rats while performing each test for maximum 48 hours after treatment. The stages of oral acute toxicity test were as follow. The rats were fasted overnight and then the body weight was measured. In the first test, the first rat was given a dose limit of 2000 mg/kgbw of ethanolic extract of Dayak onion leaves and let it rest for 48 hours. If the first animal lives, then 4 test animals were given the same dose. If 3 or more die then the dose of the main test was processed. If 3 or more live the observation was continued till 14 days. The treatment was continued to the limit test dose 5000, if the number of animals that live more was than 3 in the test of the test dose limit of 2000 mg/kgbw. In the test limit test dose 5000, if the number of animals live is more than 3 then the value of LD_{50} > 5000 mg/kgbw. If the number of live animals is less than 3 then it is continued to the main test test.

Acute toxicity observation parameters of this study include LD_{50} values, observation of toxic symptoms, changes in body weight. Toxic symptoms were analyzed qualitatively during testing based on the criteria set by OECD 425 including platform, straub, piloerection, ptiosis, corneal reflex, pineal reflex, lacrimation, catalepsy, body posture, hanging, *retablismen*, flexion, hafner, mortality, grooming, defecation, urination, breathing, salivation, vocalization, tremors, seizures and writhing. Body weight changes was quantitatively analyzed from weighing on the first day of test preparation until the 14^{th} day of observation.

The first observation of toxic symptoms was performed individually at 0, 0.5, 1 to 2 hours after administration. Then the observations were performed again at the 24^{th} hour and 14^{th} day unless the animals die . Finally observation was stopped.

LD_{50} assessment. LD_{50} value was assessed statistically including maximum likelihood method. All the doses and animal test responses were used for input to *AOT425StatPgm* software to obtain the value LD_{50} estimation.

RESULTS AND DISCUSSION

Extraction. Maceration method provided 35.7 gram of extract with yield value of 17.85%.

Extract standardization

Specific parameter. Organoleptic parameters of the extract were determined with senses to give the first impression of extract objectively such as form, colour, taste, and smell. The result showed that ethanolic extract of Dayak onion leaves was condense, blackish brown, unique smell, and bitter taste.

The assessment of soluble compound content within certain solvent (water/ethanol) aims to give preliminary depiction of compound number that can be extracted within the solvent (water/ethanol)[11]. The required amount of soluble compound within certain solvent (water/ethanol) is = 18 for water solvent and = 12.5 for ethanol solvent[12]. The test result of water soluble compound of Dayak onion leaves as much as 20.2% (w/w).

Flavonoid total assay is performed through spectrophotometry UV-Vis using quercetin comparator. Flavonoid determination principle is based on colorimetric reaction of $AlCl_3$ with keto group on C-4 atom as well as hydroxy group on C-3 or C-4 atom which neighbour of flavone and flavonol which could be analyzed in visible area. Calibration curve making is using quercetin as comparator, which quercetin is flavonoid in flavonol class that has keto group on C-4 atom and has hydroxy group on C-3 and C-5 atom. They neighbour to flavone and flavonol. Absorbance of standard liquid is measured on 510 nm wave length using spectrophotometry Uv-Vis. Standard curve is obtained from the relation between quercetin concentration (mg/L) and absorbance[13]. The result shows that total flavonoid of quercetin equivalent on ethanolic extract of Dayak onion leaves as much as 8.08% (b/b). Extract specific parameter test result can be seen on table 1.

Table 1. Test Result of EEEaL Specific Standard Parameter

No	Parameter	Result
1	Organoleptic	Colour : blackish brown Form : condense Taste : bitter Smell : unique
2	Total Equivalent Flavonoid Content	8.08% b/b
3	Active Compound Assay	(+) flavonoid
4	Water soluble compound content	20.2 % (w/w)

Active compound assay using KLT method which the compound used to standardization of Dayak onion leaves ethanolic extract is quercetin. Quercetin compound identification in Dayak onion leaves ethanolic extract using KLT method with specific reactor (vanillin $H2SO_4$). Obtained result respectively show that greenish brown and brick red. Furthermore, KLT result is compared to quercetin compound (comparator compound). KLT result can be seen on Figure 1.

Figure 1. KLT Profile of ethanolic extract of Dayak onion leaves (E) and quersetin (Q) after sprayed by sulphate vanilin for spot apparent flavonoid c luster

Non-specific parameters. Standardization and non-specific aspect are led to permitted maximum limit of dangerous material contain in the extract. Test result of non-specific parameters can be seen on Table 2.

Much amount of water content of extract is risky for it easily decay and fungus growing[14]. In this research, obtained water content of extract as much as 19.55%. Obtained extract is condensed extract type because according to the literature, water content range that required as condensed extract type approximately 5-30%.

Ash content is parameter that shows of minerals content within the extract. On the ash content assay, all the organic compound will be oxidised into their elements, while the minerals will be turned into their oxide form. Obtained ash content of Dayak onion leaves ethanolic extract as much as 11.83% (w/w). Total ash content within Dayak onion extract indicates that gained extract through maceration process contents a lot of minerals.

Lead content assay within extract is useful to ensure that the extract does not contain lead that surpass the permissioned limit due to its toxic character for human. Research result shows that lead content on ethanolic extract of Dayak onion leaves that assessed through atomic absorption spectrometry is < 0.02 mg/kgbw. Therefore ethanolic extract of Dayak onion leaves is qualified natural medicine standardization parameter. Maximum limit of lead (Pb) contaminant according to Dirjen POM No 03725/B/SK/VII/89 decree is 10 mg/kg.[15]

Bacterial contaminant test include as one of the tests of extract purity requirement. This test comprises determination of microorganism number which permitted and aiming to show that there is no certain bacteria within the extract. Indonesia National Standard (SNI) 19-2897-1992 which designated by the government states that microbes content on processed food product, < 10^6 CFU/gram for bacterial class, and < 10^4 CFU/gram for mold/yeast class. Bacterial contaminant number data of Dayak onion leaves extract as much as 3,5x10^6 CFU/gram. This number is quite high from required limit number, which is < 10^6 CFU/ml. While total plate number assessing of extract's mold as much as 1510 CFU/g, considered as over the maximum limit mold contaminant, which is < 10^4 CFU/ml. This contamination could happen during sample process until it become extract.

Acute toxicity test. Acute oral toxicity test is one of the pre-clinic test that aims to see toxic effect occured in short period, through single administration each oral or through repeated doses on 24 hours interval.[9] Animal tests death data states with Lethal Doses 50 (LD_{50}) which is the parameter on acute toxicity test.[10] LD_{50} assay is the first step to know the material safety that will be used by humans through assessing the number of doses that can cause 50% death chance on test animal after single doses administration.

Initial test to discover whether a test material has toxicity at a certain dose is done through the Up and Down Procedure (UDP) method. The UDP method is used in acute toxicity test because it is reliable and efficient in assessing LD_{50} values. This method can accurately estimate hazard classification and minimize test animals. The dosage used in this study is the limit test according to OECD 425 guidelines. There are two limit tests, they are 2000 mg/kgbw and 5000 mg/kgbw. Acute toxicity test of ethanolic extract of Dayak onion leaves with doses 2000 mg/KgBw limit test aims to discover whether the ethanolic extract of Dayak onion leaves has LD_{50} over or under 2000 mg/kgBw, while doses 5000 mg/kgBB limit test aims to discover whether the ethanolic extract of Dayak onion leaves has LD_{50} over or under 5000 mg/kgBB. LD_{50} value is obtained through data processing with *AOT425 StatPgm* software. Limit test results using doses 2000 mg/kgbw and doses 5000 mg/kgbw are shown on Figure 2 and 3.

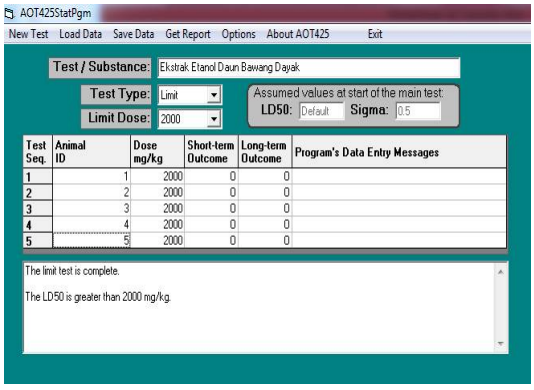

Figure 2. Limit Test Result with Doses 2000 mg/kg bw

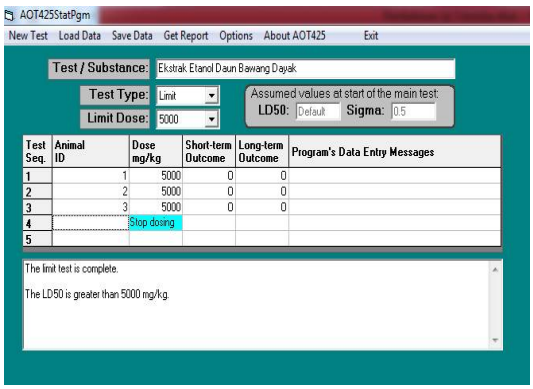

Figure 3. Limit Test Result with Doses 5000 mg/kg bw

Table 2. Test Result of EEEaL Non-specific Standard.

No	Parameter	Result
1	Water content	19.55 %
2	Ash content	11.83 % b/b
3	Heavy metal contaminant	
	- Lead	<0.02 mg/Kg
	- Bacterial total assay	$3{,}5x10^6$ CFU/gram
	- Mold total assay	1510 CFU/gram

Based on the test it can be concluded that the LD_{50} value of ethanolic extract of Dayak onion leaves is more than 5000 mg/kgbw.

Toxicity signs are observed through behaviour test starts from 0, 0.5, 1 and 24 hr. This observation tends to see the activity and normal condition of animal tests before and after doses administration. The observed behaviour test including platform, straub, piloerection, ptosis, corneal reflex, pineal reflex, lacrimation, catalepsy, gesture, hanging, *retablismen*, flexion, hafner, mortality, grooming, defecation, urination, respiration, salivary, vocalization, tremor, cramping, and writhing. If there any behaviour changing on animal tests after preparation administration, therefore it could be predicted that the pharmacology effect upon the extract apparent. Observation result of behaviour test on doses 2000 mg/kgbw limit test is shown on Table 3 and 4. In general, behavior test toward rats at the 2000 and 5000 mg/kgbw limit test show normal results.

Table 3. Test Result of Rats Behaviour on Doses 2000 mg/kgbw

Observed Effect		Rat 1					Rat 2					Rat 3					Rat 4					Rat 5				
		0	½	1	2	24	0	½	1	2	24	0	½	1	2	24	0	½	1	2	24	0	½	1	2	24
Platform		6	4	1	1	2	8	5	2	3	2	1	8	4	4	5	-	-	-	-	6	4	3	3	6	5
Motoric activity	Up	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Down	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Quiet	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Straub		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ptosis		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinela reflex		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cornea reflex		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lacrimation		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalepsy		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gesture	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Abnormal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hanging		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Retablismen		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flexion		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hafner		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mortality		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grooming		-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	2	3	4	-	-	2	5	-	-
Defecation		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urination		-	-	1	-	-	2	-	-	1	-	1	-	-	-	-	2	2	1	1	-	-	-	-	-	-
Respiration	Fast	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Stertorous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salivary		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vocalization		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremor		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cramping		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Writhing		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4. Test Result of Rats Behaviour on Doses 5000 mg/kgbw

Observed Effect		Rat 1					Rat 2					Rat 3				
		0	½	1	2	24	0	½	1	2	24	0	½	1	2	24
Platform		-	-	-	1	-	-	1	-	-	-	3	-	3	1	1
Motoric activity	Up	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Down	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Quiet	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Straub		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Piloerection		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ptosis		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinela reflex		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cornea reflex		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lacrimation		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalepsy		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gesture	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Abnormal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hanging		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Retablismen		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flexion		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hafner		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mortality		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grooming		-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Defecation		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urination		-	-	1	-	-	2	-	-	1	-	1	-	-	-	-
Respiration	Fast	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Stertorous	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salivary		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vocalization		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremor		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cramping		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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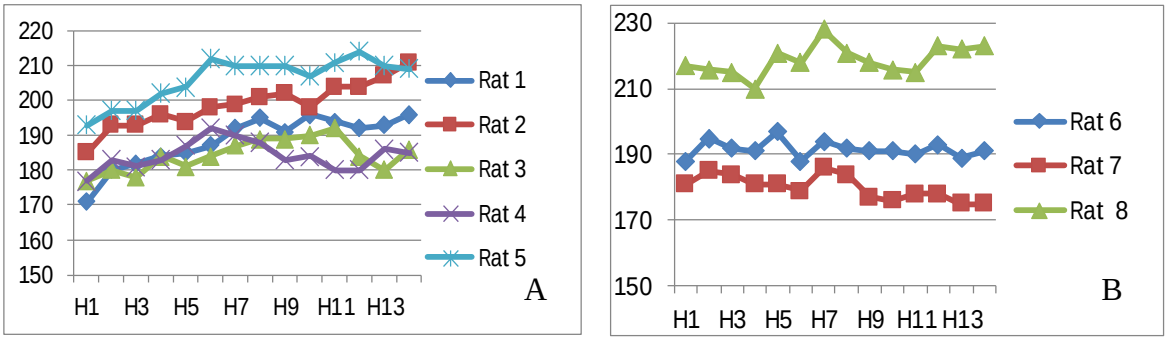

Figure 4 . Body Weight Changing Charts on Doses 2000 mg/kg bw (A) and Body Weight Changing Charts on Doses 5000 mg/kg bw (B)

Body weight changing is assessed through rats weighing on the first day until the fourteenth day after the administration of single doses orally. Rats' body weight changing data is presented in chart to observe the test agent effect on body weight condition. Body weight observation performed for 14 days shows that ethanolic extract of Dayak onion leaves administered to the rats on doses 2000 mg/kgbw lead to some rats have gained weight and some rats haven't. In rats given ethanolic extract of Dayak leaves with dose as much as 5000 mg/kgw also occured the same thing. Thus it is proven that the ethanolic extract of Dayak onion leaves at doses of 2000 and 5000 mg/kgBW generally do not cause weight loss. The results of weight observation data of rats which given doses of 2000 and 5000 mg/kgBW are shown in Figure 4.

CONCLUSION

LD_{50} value of ethanolic extract of Dayak onion leaves found to be more than 5000 mg/kgBW. According to Loomis classification, lethal doses of ethanolic extract of Dayak onion leaves considers as practically non-toxic category. The high LD_{50} value proves that ethanolic extract of Dayak onion leaves could be the safe therapy agent.

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