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Original Research

Characterization And Acute Toxicity Test Of Black Garlic Ethanol Exctract Based On OECD

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ABSTRACT

Background: *Garlic (Allium sativum, L) was used empirically by the ancestors as a useful plant for hypercholesterolemia. The processed garlic product, namely black garlic, has gone through an optimal heating process at a temperature of 70°C for 21 days. Black garlic can become a standardized herb if it has been through characterization and toxicity tests.*

Methods: *The characterization test is known by measuring several general standard parameters of Indonesian extracts. Acute toxicity test was carried out by dividing 5 treatment groups namely negative control, group I (5 mg/kg BW), group II (50 mg/kg BW), group III (300 mg/kg BW), group IV (2000 mg/kg BW).*

Results: *The results showed that the standardization of black garlic extract has a drying shrinkage content of 7.24%, a water content of 8.8%, a total ash content of 4.79%, an acid insoluble ash content of 1.52%, a water soluble extract content of 7.47% and an ethanol soluble extract content of 9.3% which is still into the Indonesian herbal pharmacopoeia standard and the administration of black garlic ethanol extract caused mild toxic symptoms during the acute toxicity test and obtained LD_{50} values > 2000 mg/kg BW.*

Conclusion: *Characterization of black garlic extract according to standardization parameters of Indonesian plant extracts and administration of ethanolic extract of black garlic has an LD_{50} value > 2000 mg/kg BW which is included in category 5 in the OECD.*

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INTRODUCTION

Plants native to Indonesia can be consumed as herbal medicines if they have been proven to be safe, efficacious and of good quality (RI, 2016). Distribution of traditional herbal medicines in Indonesia in the form of Jamu, Standardized Herbal Medicines (OHT) and Phytopharmaceuticals that require different supporting evidence (empirical,

non-clinical and/or clinical) and different standardization of raw material. The three groups are not allowed to contain medicinal chemicals.

Utilization of these herbal medicines that can be used in health services must be accounted for in terms of safety and efficacy / effectiveness with supporting evidence in accordance with claims (BPOM, 2014). One of indonesia's native plants commonly used as herbal medicine is garlic (*Allium sativum*, L). One of the processed garlic products that has been consumed by people in Asia (Japan, China, Korea, Thailand) for the last 10 years is black garlic. Black garlic is fresh garlic (*Allium sativum*, L) that has undergone a fermentation process for a certain period of time at high temperature and humidity (Kimura et al., 2017). Black garlic was first developed in Japan in 1999 by Kamimura who was granted a patent in the manufacture of black garlic from the Japan Patent Office (Sasaki, 2015).

Toxicity test is a test to detect the toxic effect of a substance and to obtain typical dose-response data from the test preparation. The toxic effect of a preparation can be known after administration of the tested dose using acute, subchronic and chronic toxicity tests. Acute toxicity test is a test to determine the toxic effect that appears in a certain period after administration of the test preparation in a single dose, or repeated doses in a 24-hour period (BPOM, 2014).

The results of the acute toxicity test will be evaluated based on the hazard criteria of the GHS (Globally Harmonized Classification System for Chemical Substances and Mixtures) listed in the Thirteenth Addendum to The OECD (Organisation for Economic Co-operation and Development, 2002) Guidelines for the Testing of Chemicals is presented ini table 1:

Table 1. Toxicity Range Categories Based on LD_{50} Value (BPOM, 2014)

Category	Value Range
1	LD_{50} < 5 mg/kg BW
2	LD_{50} > 5 mg/kg BW < 50 mg/kg BW
3	LD_{50} > 50 mg/kg BW < 300 mg/kg BW
4	LD_{50} >300 mg/kg BW < 2000 mg/kg BW
5	LD_{50} >2000 mg/kg BW <5000 mg/kg BW

Research on black garlic as an antitumor makes Japanese people interested in processing garlic into black garlic by using a rice heater (Sasaki, 2015). Black garlic can reduce the volume and weight of human gastric cancer cells SGC-7901 (Kimura et al., 2017). Black garlic can also inhibit the growth of HT29 cells through apoptosis and cell cycle arrest through the phosphatidylinositol 3-kinase protein kinase B (PI3KAkt) signal transduction pathway (Dong et al., 2014).

Black garlic has anti-obesity activity by giving rats black garlic for 5 weeks to reduce body weight, fat, triglycerides and increase HDL levels in the blood (Ha & Kim, 2015). Black garlic as an antioxidant by reducing thiobarbituric acid and increasing the activity of superoxide dismutase and glutathione peroxidase enzymes (Jeong et al., 2016). Black garlic as antihyperlipidemia by lowering TG (triglycerides), LDL (Low Density Lipoprotein), serum apo B and increasing HDL (High Density Lipoprotein) in the blood (Jung et al., 2014). Black garlic as an anticancer in human leukemia U937 cells. Black garlic lanang (single) did not cause death in mice at a dose of 10 mg/20 g BW, 20 mg/20 g BW and 40 mg/20 g BW, so the LD_{50} is unknown (Nuristika, 2018).

The study showed that the water content of garlic after heating on day 0 was 58.84% for 18 days to black garlic decreased to 52.90% water content (Miladulhaq, 2018). Based on several studies of black garlic above, it is necessary to conduct a characterization test of black garlic extract and acute toxicity test of black garlic extract as initial research in herbal medicine.

MATERIALS AND METHOD

The research method uses experimental methods, namely research that looks for the effect of certain variables on other variables strictly on the experimental group in various treatments and compares it with the control group on mice in vivo. The research was carried out through laboratory experimental stages in the form of collection, processing, determination of garlic plants, extraction of black garlic simplicia with 70% ethanol, characterization through the determination of specific and non-specific parameters and acute toxicity testing of black garlic ethanol extract in female white mice of the CBS-Swiss strain.

The research materials included garlic obtained from Lembang-West Java. All other chemicals and reagents were sourced commercially: ethanol from Merck (Germany), Pulvis Gummi Arabicum from J. Trading Co. Ltd. (Thailand). Research tools include thermostatic water bath (China), Rotary Evaporator RE 100-S Dlab (China).

The prasman leaves used in this research were obtained from three different regions, namely, Bogor, Sragen, and Cikarang. All other chemicals and reagents were sourced commercially: Rutin from Sigma-Aldrich (Singapore), ethanol from Merck (Germany), n-hexane from Merck (Germany), ethyl acetate from Merck (Germany), methanol from Merck (Germany), formic acid from Merck (Germany), and acetic acid from Merck (Germany)

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The time of this research was 4 months (February – May 2019). Research at the Phytochemistry and Pharmacognosy Laboratory, Faculty of Pharmacy, University of Padjadjaran, Laboratory of Pharmacognosy and Phytochemistry of STIKes Santo Borromeus, Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, University of Padjadjaran.

The test animals used in the acute oral toxicity test were female white mice of the CBS-Swiss strain, 3-4 months old, weight of mice 20-40 g. The mice used in this study were 25 female mice. These test animals were obtained from the Inter-University Research Center, Bandung Institute of Technology (PPAU-ITB). The test animals were adapted for 7 days and black garlic was administered orally in various doses. Safety test by acute toxicity test with 5 treatment groups namely Negative Control, Group I (5 mg/kg BW), Group II (50 mg/kg BW), Group III (300 mg/kg BW), Group IV (2000 mg /kg body weight). The results of the acute oral toxicity test in mice were seen by obtaining the LD_{50} value of the sample (BPOM, 2014).

The study design for the acute oral toxicity test was a randomized block design, in which the test animals were selected at random and had the same opportunity to be given treatment.

Analysis of research data was carried out by observing specific and non-specific parameters of the test sample in accordance with the guidelines for the parameters of general Indonesian plant extracts and acute toxicity tests were carried out by observing the test animals individually at least in the first 30 minutes after the administration of the test preparation, and periodically every 4 hours for the first 24 hours, and once a day thereafter for 14 days. Confirmation of the presence of compound markers in black garlic is done by calculating the Rf (Rf) Retardation Factor. The Rf value obtained from the comparison of the distance traveled by the component divided by the distance traveled by the solvent.

The results of acute toxicity in animals can be tested based on the hazard criteria of the GHS (Globally Harmonized Classification System for Chemical Substances and Mixtures) contained in the Thirteenth Addendum to The OECD Guidelines for The Testing of Chemicals (2001).

RESULTS

Processing of garlic into black garlic is done by wrapping 1000 g of garlic bulbs in aluminum foil and warming all the time in a rice cooker at ±70°C for 21 days with constant weight. Heating at a temperature of ±70°C aims to prevent the important compounds contained in garlic bulbs from being damaged. Black garlic is then cooled. When fresh garlic is heated, it turns black, has a jelly-like texture and is sticky, has a sweeter and sour taste than fresh garlic.

(a) (b) (c)

Figure 1. The Process of Making Garlic Into Black Garlic

Description: (a) Fresh garlic; (b) Garlic wrapped in aluminum foil; (c) Black garlic after being heated for 21 days at a temperature of ±70^{o}C

Extraction was carried out using the maceration method with 1000 g of black garlic and 3.580 L of 70% ethanol macerated during 5x24 hours (Marjoni, 2016). The maceration method was chosen because it is an effective method with the aim of extracting a lot of metabolites. The solvent used is 70% ethanol. Ethanol is used as a solvent because ethanol is a versatile solvent that is good for preliminary extraction and can dissolve almost all secondary metabolites contained in simplicia and is safe. The extract obtained from the maceration was the extract obtained from the maceration was 495.84 g. Then calculated the yield obtained as much as 49.58%.

The following are Characterization Results of Garlic Extract Parameters.

Table 2. Drying Shrinkage Results

Cup	Drying Loss Rate	Drying Loss Rate Average (%) ± SD
1	7,24	7,24 ± 0,02
2	7,22	
3	7,26	

Table 3. Results of Water Soluble Essence Level

Var	Cup	Water Soluble Level	Average Water Soluble Content (%) ± SD
Black Garlic Extract	1	7,5	7,47 ± 0,651
	2	6,8	
	3	8,1	

Table 4. Ethanol Soluble Extraction Results

Var	Cup	Ethanol Soluble Essence Level	Average Ethanol Soluble Extract Content (%) ± SD
Black Garlic Extract	1	9,6	9,3 ± 0,361
	2	8,9	
	3	9,4	

Table 5. Total Ash Content Results

Var	Cup	Total Ash Content	Average Total Ash Content (%) ± SD
Black Garlic Extract	1	4,65	4,79 ± 0,318
	2	5,15	
	3	4,56	

Table 6. Result of Acid Insoluble Ash Content

Var	Cup	Acid Insoluble Ash Content	Average Acid Insoluble Ash Content (%) ± SD
Black Garlic Extract	1	1,40	1,52 ± 0,225
	2	1,78	
	3	1,38	

Table 7. Water Content Results

Cup	Water content	Average Water Content ± SD
1	8,9	8,8 ± 0,513
2	9,2	
3	8,2	

The standardized value of the quality parameter of the black garlic extract meets the standard value in accordance with the Indonesian Herbal Pharmacopoeia and the Indonesian Ministry of Health for the drying shrinkage value ≤ 10%, water content ≤ 10%, total ash content ≤ 9.06%, acid insoluble ash content ± 1%, water soluble extract content ≥ 5.0%, ethanol soluble extract content ≥ 4%.

Table 8. Phytochemical Screening Results

Secondary Metabolites	Reagents	Result
Alkaloids	Dragendroff, Mayer, Bouchardat	+
Flavonoids	Ferri Chloride	+
Saponins	Foam test	-
Polyphenol	Iodine	+
Quinone	Sulphuric acid	-
Tannins	Gelatin	-
Monoterpenoids / Sesquiterpenoids	Copper acetate	+ / +
Steroids / Triterpenoids	Salkowski's	+ / +

Information:
(+) : Detected
(-) : Not detected

The results of phytochemical screening showed that in black garlic, secondary metabolites of alkaloids, flavonoids, monoterpenoids / sesquiterpenoids and steroids / triterpenoids were detected which are useful for the treatment of hypercholesterolemia.

Results of Determining the Content of S-Allyl Cysteine in Black Garlic. Black garlic simplicia was analyzed for its components by thin layer chromatography.
Stationary phase : Silica gel 60 F_{254}
Mobile phase : Toluene:ethyl acetate (7:3)
Test solution : 5% in ethanol P
Comparison solution : *S-Allylcysteine 0.1% in ethanol P*
Spotlight volume : 3 µL test solution and comparison solution
Detection : UV light 254 nm

Figure 2. Chromatogram. (S) Black garlic extraxt; (P) s-allylcysteine

Comparative Rf S-allylcysteine 0.59:
Rf 1 = 0,19
Rf 2 = 0,41
Rf 3 = 0,59
Rf 4 = 0,85

Results of Weighing Test Animals (Fixed Dose Method)

Table 9. Body Weight Results of Mice

Dosage Group Female Mice (mg/kg BW)	The average weight of female mice (grams) on day						
	1	2	3	4	5	6	7
5	24,5	24,5	25	25,75	26	26,25	27,75
50	24	24	25,75	26,3	26,8	27,75	28,5
300	23,75	23,5	25	25,9	26,2	26,75	28,25
2000	23	23,5	24	24,5	24,75	26,75	27
Negative Control (feed only)	23	23,5	25,85	26,45	26,5	26,95	27,25

Dosage Group Female Mice (mg/kg BW)	The average weight of female mice (grams) on day						
	8	9	10	11	12	13	14
5	27,56	27,35	27,67	26,96	26,86	26,55	26,75
50	28,57	28,9	28,5	28,1	28,5	27,75	27,25
300	28,25	28,25	28,58	29,45	29,56	29,35	29,25
2000	27,56	27,75	27,98	28,35	28,67	28,78	28,75
Negative Control (feed only)	27,35	27,56	27,95	28,36	28,68	29,56	29,75

Table 10. Results of Observation of Test Animal Mortality in Preliminary Test

Amount Mice	Dosage Group (mg/kg BW)	Amount Death	% death
5	5	0	0
5	50	0	0
5	300	0	0
5	2000	0	0
5	Negative Control (feed only)	0	0

Table 11. Results of Observation of Signs and Symptoms of Toxic Effects for 14 Days Negative Control Group and Treatment Group

Observation	Signs of Toxicity	Negative Control	Treatment Group
Central nerve system	Motor Activity	Normal	Normal
	Hanging activity	Normal	Normal
	Retablism	-	-
	Body posture	Normal	Normal
	Tremor effect	-	-
	Convulsions	-	-
	(seizures)	-	-
	Straub	-	-
	Catalepsy	-	-
	Sedative	-	Normal
	Flexion	Normal	Normal
	Hafner	Normal	

Observation	Signs of Toxicity	Negative Control	Treatment Group
	Pineal Reflex	Normal	Normal
	Corneal Reflex	-	-
	Ptosis	Normal	Normal
	Grooming	-	-
	Respiration		
Autonomic Nervous System	Piloerection effect	Normal	Normal
	Salivation	-	-
	Lacrimation	-	-
	Abnormal urination	-	-
	Diarrhea	-	-

The multilevel doses given in this fixed dose method are 5, 50, 300, 2000 kg/kg BW. The first test was carried out by giving a dose of 5 mg/kg BW and observed toxic symptoms and death for 24 hours in female mice. When death does not occur, then continue with a dose level of up to 2000 mg/kg BW. The results showed that the administration of doses up to 2000 mg/kg BW did not result in death and mild toxic symptoms.

Pharmacological screening was carried out at 30 minutes, 4, 8, 12, and the first 24 hours and after that for 14 days. Observations made consisted of observations of the central nervous system, namely motor activity, hanging activity, retablisment, posture, tremor effects, convulsions, straub, catalepsy, sedative, flexion, hafner, pineal reflex, corneal reflex, ptosis, grooming or breathing. Then, observations on the autonomic nervous system, namely the effects of piloerection, salivation, lacrimation, abnormal urination, and diarrhea.

From the results of observations for 14 days, no toxic symptoms appeared and there was no change in the behavior of the test animals, both from the observation of the central nervous system and the autonomic nervous system.

DISCUSSION

From a theoretical perspective, this research is expected to provide scientific information regarding the most appropriate level of quality in black garlic extract which can be used as Standardized Herbal Medicine. This research is seen from the practitioner's aspect, it is expected to be able to determine the safety level of black garlic extract through the LD_{50} value so that it can be consumed by the public as a reference for the treatment of hyperlipidemia.

The standardized value of the quality parameter of the black garlic extract meets the standard value in accordance with the Indonesian Herbal Pharmacopoeia and the Indonesian Ministry of Health for the drying shrinkage value ≤ 10%, water content ≤ 10%, total ash content ≤ 9.06%, acid insoluble ash content ± 1%, water soluble extract content ≥ 5.0%, ethanol soluble extract content ≥ 4%. The results for the parameters tested include drying shrinkage has a value of 7.24%, water soluble extract content of 7.47%, ethanol soluble extract content of 9.3%, total ash content of 4.79%, acid insoluble ash content of 1, 52% and 8.8% water content.

All quality parameters of black garlic ethanol extract are in accordance with the standard parameters of Indonesian medicinal plant extract quality. The Rf value

obtained from the comparison of the distance traveled by the component divided by the distance traveled by the solvent with the Rf of the test solution has 4 spots with an Rf value of 3 equal to the Rf value of the S-allylcysteine as a comparison solution, which is 0.59. This means that black garlic simplicia contains S-allylcysteine compounds.

The multilevel doses given in this fixed dose method are 5, 50, 300, 2000 kg/kg BW. The first test was carried out by giving a dose of 5 mg/kg BW and observed toxic symptoms and death for 24 hours in male and female rats. When death does not occur, then continue with a dose level of up to 2000 mg/kg BW. The results showed that the administration of doses up to 2000 mg/kg BW did not result in death and no toxic symptoms.

The administration of black garlic ethanol extract did not cause toxic symptoms during the acute toxicity test, so a dose of 2000 mg/kg BW did not cause toxicity in female mice according to the Toxicity Range Category based on the LD50 value in BPOM regulations. Pharmacological screening was carried out at 30 minutes, 4, 8, 12, and the first 24 hours and once a day, after that for 14 days. From the results of observations for 14 days, no toxic symptoms appeared and there was no change in the behavior of the test animals, both from the observation of the central nervous system and the autonomic nervous system.

CONCLUSION

The standardized value of the quality parameter of the black garlic extract meets the standard value in accordance with the Indonesian Herbal Pharmacopoeia and the Indonesian Ministry of Health for the drying hrinkage value ≤ 7.24%, water content ≤ 8.8%, total ash content ≤ 4.79%, acid insoluble ash content ± 1.52%, water soluble extract content ≥ 7.47%, ethanol soluble extract content ≥ 9.3%. Administration of black garlic ethanol extract caused mild toxic symptoms during the acute toxicity test and obtained an LD_{50} value > 2000 mg/kg BW which was included in category 5 in the OECD Guidelines for The Testing of Chemicals.

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