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Acute Toxicity Study of Anti-diarrheal Herbal Combination in Mice

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Abstract

This study was conducted to test the acute anti-diarrheal toxicity made from a combination of herbal extracts contain leaves of guava (*Psidium guajava*), turmeric (*Curcuma domestica*), fruit mojokeling (*Terminalia chebula*) and pomegranate (*Punica gratum*) peel. In vivo study was carried out on female Swiss Webster mice at the Toxicology Pharmacology Laboratory of the Institut Teknologi Bandung Pharmacy School. The five tested dose levels were the equivalent dose of 1/2 (164 mg/kg mice body weight) and one (328 mg/kg mice body weight) humans daily dose and three other doses, at 1, 2, and 5 g/kg body weight in mice and one control group. The assessment included observing animal behavior caused by toxic effect after test sample administration, if any, compared to its behavior before giving the test preparation (T0) and to the control. Observations were made carefully during the first 4 hours after test preparation administration and at T8, T24, and continued periodically every day until the endpoint of testing (H14). Changes in body weight were also monitored daily and at the endpoint, the animals were sacrificed for macroscopic examination of organs and organ index determination. The results showed no behavioral and clinical signs of toxicity were found after administering anti-diarrheal herbal combination at all testing doses. There were no animals that died during the treatment, and the macroscopic examination shows there were no abnormalities found in vital organs (lungs, heart, liver, kidney, stomach) after administration of anti-diarrheal products up to a dose of 5 g/kg body weight of mice. Taking all these into account, it can be concluded that those as mentioned earlier, the anti-diarrheal herbal combination is not toxic, and its lethal dose of 50 (LD50) is >5 g/kg mice body weight.

Keywords: Acute Oral Toxicity, Herbal Combination Anti-diarrheal, LD50

1. Introduction

Diarrhea is a clinical symptom of digestive tract disorders characterized by increased defecation frequency, accompanied by a change in the stool's consistency to become soft or liquid and an imbalance of body fluids and electrolytes. In Indonesia, the prevalence of diarrhea, according to the Basic Health Research of the Ministry of Health of the Republic of Indonesia (Riskesdas) 2018[1], reaches 7%. Diarrhea can be caused by many factors, including bacterial infection, food poisoning, allergic reaction and it can also occur due to stress. Based on the time course, diarrhea is also classified as acute, persistent and chronic. Acute diarrhea is defined as three or more loose bowel movements during 24 hours and also the duration is less than two weeks. Diarrhea is persistent if the duration varies from 2 to 4 weeks and chronic if it lasts over four weeks in duration[2]. It can even be classified supported by the etiology as infectious or non-infectious. Non-infectious diarrhea, e.g., irritable bowel syndrome, results in a posh interaction of immune and neuronal factors. The mechanisms of diarrhea caused by various pathogens are

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often classified as inflammatory or non-inflammatory[3]. Anti-diarrheal will be classified into several groups: antimotility, adsorbents, drugs that change electrolytes, and fluid transportation[4]. Loperamide HCl could be pretty common to use for diarrhea. However, it also has to be used in precaution due to its side effects, which will cause stomach upset, nausea, vomiting, drowsiness, dizziness, and dry mouth[5].

Medication side effects often become the reason people take herbal medicine as an alternative treatment. Indonesia is well known as a country with very high biodiversity and many of Indonesia's herbals have been used for traditional medicine. Some plants are traditionally used as anti-diarrhea, such as turmeric, guava leaves, bay leaves, pomegranate peel, mojokeling fruit, ginger and many more. These plants have certain active substances that play a role in suppressing diarrhea. Compared to chemicals, the traditional herbal medication known as "jamu" has several advantages, which are more economical, has less and more tolerable side effects and has more benefits due to its various active substance containing thus able to have a multiple targeting treatment[6].

The anti-diarrheal herbals used in this paper consist of a combination of herbal guava leaves (*Psidium guajava*), turmeric (*Curcuma domestica*), mojokeling fruit (*Terminalia chebula*), and pomegranate peel (*Punica gratum*). In this herbal combination, there were tannins, alkaloids and flavonoids, which are thought to reduce the frequency of defecation[7]. Guava leaves extract contained in this herbal medicine contains quercetin that had properties as chelating spasmolytic effects that shrink the intestine so that intestinal peristalsis is reduced and suppresses the diarrhea[8]. Furthermore, tannins and flavonoids increase colonic water and electrolyte reabsorption and while other phytochemicals such as phenolic and alkaloid compounds act as intestinal motility inhibitor[9]. Flavonoid content in plant extracts reported inhibiting autacoids and prostaglandins' release, thereby inhibiting motility and secretion. Flavonoids are thought to also play a role in inhibiting enzymes prostaglandin synthase, cycloxygenase, and lypoxygenase[10], which may mainly contribute to anti-diarrhea activity due to their role in the modulation of prostaglandins and leukotrienes. These agents were involved in the pathophysiology of diarrhea.

Furthermore, using herbals as a complementary treatment should also be supported with scientific evidence to support its efficacy claims and safety assurance. To ensure the combination's safety as one of diarrhea treatment, toxicity assessment should be done through preclinical testing. The acute toxicity study aims to provide information on the safety of using a single dose of this herbal combination. In this study, the acute toxicity of anti-diarrheal herbal combination was tested on female Swiss Webster mice obtained from the Animal Laboratory of the Institut Teknologi Bandung (ITB) School of Pharmacy. The test guidelines used in the study refer to the toxicity test guidelines issued by the Indonesian National Agency of Drug and Food Control (Badan Pengawas Obat dan Makanan Republik Indonesia: BPOM RI)[11] in Peraturan Kepala BPOM no. 17/2014 and the Organisation for Economic Co-operation and Development (OECD)[12]. The anti-diarrheal herbal combination is given orally in a single dose with five doses with two doses equal to the recommended dose in humans, while the other three doses are 1, 2, and 5 g/kg mice body weight, respectively. Following the test guidelines, observation of the effects on the mice behaviors was carried out carefully on the day of the administration and then continued every day for 14 executive days. This study was conducted after obtaining approval from the Commission of Ethics for the Use of Experimental Animals at ITB number: 06 / KEPHP-ITB / 06-2017, June 9, 2017.

2. Material and Methods

2.1 Materials and Apparatus

The materials used in this study are anti-diarrheal herbal combination obtained from PT SOHO Industri Pharmasi in capsules containing active ingredients of guava (*Psidium guajava*) leaves extract 240 mg, turmeric (*Curcuma domestica*), rhizome extract 204 mg, mojokeling (*Terminalia chebula*) fruit extract 84 mg and pomegranate (*Punica gratum*) peel extract 72 mg, CMC sodium and distilled water. The instruments and apparatus used for the study are a set of the acute toxicity test kit, surgical instruments, oral syringes for mice, mortars and pestles, electric stoves and laboratory glassware.

2.2 Test Animal

The test animals were female Swiss Webster mice aged 6-8 weeks obtained from the Animal Laboratory of the Pharmacy School of ITB. Animals are kept in a room with average air circulation with 12 hours of light and 12 hours of darkness, controlled temperature and 50-70% humidity. Standard feed and drinking water are given in excess[11].

2.3 Preparation of Test Sample

Initially, the average weight of the capsule contents is determined for calculating further test dose. Each capsule of the anti-diarrhea herbal combination was weighed separately with a total of 10 capsules and then the average

weight per capsule was calculated. The capsules were then crushed and homogenized, followed by weighing a certain amount of anti-diarrheal herbal combination powder according to the test dose in Table 1. All test doses are suspended in CMC sodium 0.3% w/v in distilled water.

2.4 Determination of Dosage

The dose recommendation for the anti-diarrheal herbal combination is 2x2 capsules per day. From the daily dose recommendation and the calculated average weight per capsule (629.7 mg), five doses are derived from being tested in this toxicity study. Two doses of which are equivalent to half the daily dose recommendation - 2 capsules (163.72 mg/kg body weight in mice) and its daily dose recommendation - 4 capsules (327.44 mg/kg body weight in mice). The other three doses are 1000, 2000 and 5000 mg/kg body weight of mice (Table 1). The last three test doses are selected according to the test guidelines, where these doses can provide an overview of an extract's safety (OECD / BPOM RI). The dose equivalent from humans to animals (mice) was calculated using a conversion factor[13].

Table 1. Test dose calculation

Group	Test dose Equivalent to	The test dose becomes
D1	2 capsules for use in humans	Human dosage (2 capsules) = 2x629.7mg = 1,259.4 mg The dose for mice 20 g = 1.259.3 x 0.0026 = 3.27 mg (= 163.72 mg/kg body weight mice)
D2	4 capsules for use in humans	Human dosage (4 capsules) = 4x629.7mg = 2,518.8 mg The dose for mice 20g = 2.518.8 x 0.0026 = 6.55 mg (= 327.44 mg/kg body weight mice)
D3	1 g/kg body weight of mice	1g/kg body weight (equivalent to 12.22 capsules)
D4	2 g/kg body weight of mice	2g/kg body weight (equivalent to 24.43 capsules)
D5	5 g/kg body weight of mice	5g/kg body weight (equivalent to 61.2 capsules)

2.5 Treatment

The mice were fasted to eat for 4 hours before the treatment. On the day of testing, the mice were given the test material in the form of the anti-diarrhea suspension in CMC sodium 0.3% w/v orally with a volume of 1 mL per 20 g of mice according to the test dose: 1 dose per test group. The control group was only given a carrier (CMC sodium 0.3% w/v) with the same administration volume.

2.6 Observation

Careful observations were made at T0 (before any treatment was given), T1, T2, T4, T8, and T24 hours after oral administration. The observation was continued two times daily in the morning and evening for 14 executive days after oral administration. Observations were made on each test animal individually.

The observation parameters include behavior and toxic reactions (time of appearance, duration and severity of effects), death (cause and time of death). The toxic reaction observation includes somatomotor activity, corneal, pineal reflexes, flexion, urine profile (volume, color, frequency); frequency and consistency of stool, changes in respiratory rate and heart rate, dilation of blood vessels, relaxation and muscle contraction, Haffner's phenomenon, Straub effect, tremor, ptosis, lacrimation, piloerection, writhing (stretching), righting reflex, catalepsy, posture and motoric activity. Those observation parameters on toxic reaction reflect the test material's effect on the nervous, respiratory, cardiovascular, digestive, urinary, locomotor and integumentary systems. According to the OECD guideline for testing chemical toxicity, particular attention is also paid to the appearance of tremors, convulsions, salivation, diarrhea, vomiting, lethargy, depression, and coma.

Changes in body weight of test animals were also monitored every day until the endpoint of the study. The obtained observation results were also compared to the control animals group. At the endpoint, the animal was ethically sacrificed according to the Ethical Commission's recommendations on the Use of Experimental. All of the individual organs such as the liver, kidney, lung, heart, spleen, adrenal glands and reproductive organs of female mice were observed macroscopically, and their appearance was compared between both treated and control groups. The relative organ weight of each animal was then calculated

as follows. Relative organ weight: (absolute organ weight/ body weight × 100%) of mice on the day of sacrifice[14].

2.7 Analysis and Evaluation of Results

The obtained organ index data from each group were calculated, an average from each group with its standard deviation was then compared to the controls and statistical significance was determined. The robust statistical method used is the Least Significant Difference alpha (LSDα).

3. Results

3.1 Animal Behavior and Toxicity Reaction Observation

In general, no significant toxic effects were observed after administration of the five dose levels. The only salient changes are in motor activity and posture. However, these motor activity and posture changes occurred in all test groups and the control group. After administering a single dose of up to 5 g/kg body weight in mice, there is no Straub effect, piloerection, ptosis, catalepsy and lacrimation. The five tested dose levels of anti-diarrhea did not cause any vomiting, salivation, convulsions, lethargy and diarrhea, which are the main toxic effects parameters that should not occur in pharmaceutical preparations to be used in humans according to OECD guidelines. Body tremor was observed in 1 mouse (20%) from all the dose groups except the D4 group. Since body tremor also occurred in the control mice group, it can be concluded that the body tremor in the treatment group is not caused by the toxic effect of the anti-diarrheal herbal combination administration.

Furthermore, no changes in the locomotor system were observed from all treatment groups, shown by the mice's ability to hang and retain, which remained normal after administering an anti-diarrheal herbal combination. The ability to reverse the body writhing reflex and pineal, corneal and flexion reflexes also remained normal. Heart rate and respiration were also not affected by the anti-diarrheal herbal combination administration. Urination and defecation occurred in all test groups and in the control group and its within normal limits, which further confirm that the administration of the anti-diarrheal herbal combination did not affect the urinary and digestive systems. This anti-diarrheal herbal combination also did not have an analgesic effect, as seen from Haffner's positive response in all mice in the test group (data is not shown).

3.2 Change in Body Weight

After administration of oral anti-diarrheal herbal combination, there were changes in the bodyweight of mice. The changes were varied within and between groups, but all showed a similar pattern. In the early six days after administering the anti-diarrheal herbal combination, there was increased body weight in all groups. The H7 and H9 observations show a decrease in body weight in all groups, which then increasing until the end of the study (Table 2). Furthermore, the body weight change in all treatment groups is not statistically significant compared to the control group.

3.3 Organ Index

At the endpoint of the study, macroscopic examination and organ index determination were performed. There were no significant changes in the macroscopic of the liver, spleen, lungs, heart and reproductive organs of female mice after administering the five levels of anti-diarrheal doses. The mice's organ indexes in Table 3 also show no statistical difference between the control and test groups.

Table 2. Changes in body weight

Test group	Dose (g/kg bw)	Change in body weight (%)					
		H1	H4	H7	H9	H12	H14
Control	0	4.96±2.96	3.70±3.57	-3.10±1.44	-0.04±2.01	1.25±1.38	3.66±2.62
D1	0.164	4.42±3.02	0.90±0.73	-1.26±1.91	-1.38±3.24	1.68±1.31	2.71±1.90
D2	0.328	2.90±3.49	0.93±1.13	-1.60±1.61	-1.38±1.72	2.83±2.61	4.13±1.13
D3	1	3.62±3.38	0.56±1.55	-0.83±2.16	-1.55±1.98	0.48±3.03	1.63±1.07
D4	2	2.94±2.58	2.55±1.58	-2.36±1.06	-1.71±1.38	0.18±1.22	2.46±1.51
D5	5	3.26±0.99	-0.01±4.19	-1.00±2.01	-1.16±2.01	0.23±1.52	4.93±1.95

n = 5; H1, H4, H7, H9, H12, and H14 = Time (day) 1, 4, 7, 9, 12, to 14 after giving the test substance. The body weight change in all treatment groups are not statistically significant in compare to the control group.

Table 3. Organ index

Test group	Dose (g/kg bw)	Organ Index (%)							
		Liver	Spleen	Heart	Kidney	Lung	ovary	fallopian tube	thymus gland
Control	0	5.86±0.61	0.53±0.11	0.43±0.03	1.21±0.10	0.69±0.09	0.08±0.02	0.11±0.03	0.31±0.06
D1	0.164	5.49±0.49	0.45±0.05	0.40±0.03	1.12±0.13	0.68±0.06	0.08±0.03	0.14±0.03	0.32±0.01
D2	0.328	5.39±0.21	0.46±0.10	0.36±0.05	1.14±0.06	0.81±0.16	0.08±0.02	0.13±0.04	0.39±0.21
D3	1	5.31±0.28	0.46±0.05	0.42±0.05	1.05±0.17	0.68±0.06	0.08±0.02	0.13±0.04	0.33±0.14
D4	2	5.43±0.57	0.47±0.09	0.42±0.06	1.19±0.18	0.67±0.15	0.08±0.02	0.15±0.08	0.35±0.07
D5	5	5.41±0.25	0.60±0.28	0.38±0.04	1.14±0.12	0.59±0.17	0.08±0.03	0.15±0.04	0.33±0.08

n = 5. The organ index in all treatment groups are not statistically significant in compare to the control group.

3.4 Mortality

During 14 days of observation, no mice died either in the test group given anti-diarrhea and control products (Table 4). This data shows that anti-diarrheal products made from a combination of herbs with all five dose levels given orally in a single dose are not lethal.

3.5 LD 50

In this study, single-dose administration of anti-diarrheal herbal combination up to 5 g/kg body weight of mice, equivalent to 61.2 capsules, did not cause death and is not toxic to mice. It can be concluded that the LD50 of this anti-diarrheal herbal combination is > 5 g/kg body weight of mice.

4. Discussion

Leaves of guava (*Psidium guajava*), turmeric (*Curcuma domestica*), mojokeling fruit (Terminalia chebula) and pomegranate peel (*Punica gratum*) are traditionally used as anti-diarrhea, as its single ingredient. This combination of herbal plants is expected to produce better synergy.

The anti-diarrhea effect is possibly derived from its multi-component active substance contained in the herbals. The content of tannins, alkaloids and flavonoids from each herbal play an essential role in modulating the digestive organs function, thus having pharmacological effect as anti-diarrhea. However, although the diarrhea treatment takes a short period of medication, the traditionally used combinations, as mentioned earlier, have not been appropriately assessed from the safety point of view.

The safety profile of the single component has been extensively studied. The water extract of *P. guajava* leaves has no short-term harmful effect and was found to be non-toxic to rats and mice at a dose of 5g/Kg. i.e., LD50 was more than 5 g/kg[15]. Jahromi *et al.*[16] reported that PPE studies' Toxicological potential revealed no toxic effects, clinical signs, histopathological effect in epithelial cells layer of tongue, larynx and trachea, behavioral alterations and adverse effects or mortality in BALB/c mice. The ethanolic extract of *Curcuma domestica* is harmless by acute toxicity in Rat Wistar[17]. For the study of acute toxicity, a single oral administration of the water extract *Terminalia chebula fruit* at a dose of 5,000 mg/kg body weight Spargue–Dawley Rats was performed and the results showed no signs of toxicity such as general behavior changes, morbidity, mortality, changes on gross appearance or histopathological changes of the internal organs of rats[18].

In the acute toxicity study of the anti-diarrheal herbal combination, the five doses did not affect the central and somatomotor nervous system, autonomic, respiratory, gastrointestinal, genitourinary, mucous membranes and eyes. The administration of this herbal combination also did not statistically affect the test animal's bodyweight development. Changes in body weight are indicators of drug and chemical side effects and will be significant if there is a weight loss of more than 10% of the initial weight[19]. Because the changes in body weight of mice given the five dose levels of anti-diarrheal products tested were not more than 10%, it can be concluded that this product has no significant side effects.

These results show that there is no accumulated effect of the anti-diarrheal herbal combination on the organs of mice. The organ weight is a vital indicator of physiological and

condition in humans and animals. The relative organ weight is a key to diagnose whether the organ was exposed to the injury or not. The heart, liver, kidney, spleen and lungs are the first organs suffering from the metabolic reaction caused by toxicants. This anti-diarrheal herbal combination did not induce any toxic effect on the kidneys and the other organs going by this indicator since the relative weights of the organs were not significantly different from control values[20]. The data also showed that anti-diarrheal products from a combination of herbal with all five dose levels given orally in a single dose are not lethal.

To ensure a substance's safety, a lethal dose of 50 (LD50), the dose that causes the death of 50% of the test animals, must be determined. Lethal dose 50 (LD50) is the amount of a statistically derived substance that is estimated to cause death in 50% of animals when administered by a specific route as a single dose, and the animals are observed for a certain period of time[21]. Based on the classification of the OECD 423 global harmonization system and classification, the LD50 of those anti-diarrheal products made from a combination of herbal is classified as unclassified or minimally practically non-toxic. Lethal dose 50 (LD50) of this anti-diarrheal herbal combination can be determined to be greater than 5 g/kg body weight of mice. There is no toxicologic effect from the administration of anti-diarrheal herbal combination at its recommended daily dose and up to 15 times higher dose.

5. Conclusion

No death was observed in mice treated with the anti-diarrheal herbal combination consist of leaves of guava (*Psidium guajava*), turmeric (*Curcuma domestica*), mojokeling fruit (*Terminalia chebula*), and pomegranate peel (*Punica gratum*). The observation of animal behavior and the organs index assessment shows no toxicologic effect from administering this anti-diarrheal herbal combination at its recommended daily dose up to 15 times higher. Furthermore, the lethal dose 50 (LD50) of this anti-diarrheal herbal combination can be determined to be greater than 5 g/kg body weight of mice.

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