

ANTIDIARRHEAL ACTIVITY TEST OF ETHANOLIC EXTRACT OF WHITE POMEGRANATE PEEL (*PUNICA GRANATUM L.*) IN MALE WHITE MICE (*MUS MUSCULUS*), SWISS WEBSTER LINE USING THE INTESTINAL TRANSIT METHOD

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ABSTRACT

Objective: Diarrheal disease and its complications remain a major cause of morbidity and mortality in children, especially in developing countries. It is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral, parasitic or organisms. The purpose of this study was to determine the activity and dose of white pomegranate peel (*Punica granatum L.*) ethanol extract as an antidiarrheal in white male mice using the intestinal transit method.

Methods: Mice were grouped into 5 groups: negative control (Na CMC 1 %), positive control (loperamide HCl 0.0104 mg/20 g mice BW), and pomegranate peel ethanol extract test group 1, 2, and 3 (dose of 16, 32, and 64 mg/20 g mice BW). The length of the intestine that the ink marker traversed from the pylorus to the end (which is black) was measured using a ruler.

Results: Based on statistical analysis, there were significant differences between all groups (<0.05). The highest antidiarrheal activity was in the ethanolic extract of pomegranate peel at a dose of 64 mg/20 g mice BW with an inhibition percentage of 36.44% and higher than the positive control (29.81%). The inhibition percentage was also resulted by dose 1 and 2 (12.46% and 29.53%, respectively).

Conclusion: From these results show a correlation that the higher the extract dose, the higher the antidiarrheal potential.

Keywords: White pomegranate, Antidiarrheal, Intestinal transit method

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INTRODUCTION

Diarrhea is one of the diseases that is still suffered by many people, especially in developing countries such as Indonesia. Diarrhea itself can cause electrolyte loss, dehydration, and if this continues for a long time can result in death [1]. Based on research in 2017 related to the number of all age diarrhea sufferers who were treated at health facilities, as many as 4.274.790 patients and the results of data updates showed an increase in 2018, which was 4.504.524 diarrhea sufferers or around 62.93% of the estimated diarrhea in health facilities [2]. It should also be noted that diarrhea can be caused by unhealthy food factors or foods that are processed in an unclean way so that they are contaminated with diarrhea-causing bacteria such as *Salmonella*, *Shigella* and *Campylobacter jejuni* [3]. According to ethnobotanical research, it is known that the peel of the white pomegranate is widely used as a treatment for diarrhea [4]. The results of previous studies showed that the infusion of white pomegranate peel (*Punica granatum L.*) had antidiarrheal activity in the initial parameters of diarrhea and stool weight, where the doses were 1 (8 mg/20 g), 2 (16 mg/20 g), and 3 (32 mg/20 g) has an activity to increase the onset of diarrhea and reduce stool weight [5]. Other screening results stated that the ethanolic extract of pomegranate (*Punica granatum L.*) contains secondary metabolites in the form of flavonoids, alkaloids, terpenoids, saponins and tannins [6]. In this study, the researchers wanted to prove the activity of the white pomegranate peel (*Punica granatum L.*) as antidiarrheal in white wistar mice using the intestinal transit method.

MATERIALS AND METHODS

White pomegranate peel (Panji Farm), Inamid Loperamide HCl 2 mg (PT. Nufarindo), Na-CMC technic (PT. Dipa Prasada Husada), 70% ethanol technic (PT. Dipa Prasada Husada), White Male Mice Swiss Webster strain (Allumma Mouse Farm), filter paper (PT. Brataco), Glacial CH₃COOH technic (PT. Brataco), H₂SO₄ technic (PT. Brataco), HCl technic (PT. Brataco), Magnesium powder technic (PT. Dipa Prasada Husada), FeCl₃ technic (PT. Brataco), Norite or activated carbon technic (PT. Brataco), Ether technic (PT. Dipa Prasada

Husada), NaOH technic (PT. Dipa Prasada Husada), Gelatin technic (PT. Dipa Prada Husada), Vanillin technic (PT. Dipa Prada Husada), N-Heksan technic (PT. Dipa Prada Husada), distilled water (STIKes BTH). Spluit (OneMed Health Care), oral probe (Gavage), analytical balance (Ohaus), digital balance (Excellent), stir bar, spatel, watch glass, dropper, beaker (pyrex), measuring cup (pyrex), measuring flask (pyrex), test tube (pyrex), macerator, rotary evaporator (IKA), steam cup, spirit lamp, tripod, asbestos gauze, water bath (Memert), blender (Philips), sieve, laptop (Acer), cell phone, gloves (OneMed Health Care), mask (OneMed Health Care), ruler. Fresh white pomegranate peel (*Punica granatum L.*) was obtained from the Panji Plantation of Probolinggo City, East Java.

Plant determination

This determination was carried out at the Jatinangor Herbarium, Plant Taxonomy Laboratory, Department of Biology, FMIPA, Padjadjaran University, Jatinangor, to ensure the identity of the white pomegranate (*Punica granatum L.*), which was used as the sample.

Research ethics

The research was carried out in accordance with the protocol standard of the Research Ethics Commission of STIKes Bakti Tunas Husada Tasikmalaya, No.001/kepk-bth/II/2021.

Acclimatization

Acclimatization is the maintenance of experimental animals with the aim of adapting to a new environment. Acclimatization was carried out in the Pharmacology laboratory of STIKes BTH Tasikmalaya for one week. The experimental animals used were male white wistar mice with an average weight of 20-30 grams with an average age of 2-3 mo. if there are sick or dead experimental animals, or weight loss >10%, it is excluded from this study. Experimental animals were obtained from the Allumma Mouse Farm [7].

Sample preparation

The white pomegranate peel simplicia that had been collected was first carried out by wet sorting from dirt, then washed with running

water until no dirt remained. Furthermore, chopped to obtain small pieces so as to facilitate the drying process. The skin of the white pomegranate that has been cleaned and chopped, then dried by drying in direct sunlight, then sorted dry from foreign materials that are still attached to the dried white pomegranate skin. Next, the dried simplicia skin of the white pomegranate is then blended or ground until a fine simplicia powder is obtained [8].

Extraction

The dried powder of white pomegranate peel was weighed as much as 400 grams and then macerated with 70% ethanol solvent. Maceration was carried out at room temperature for 3x24 h and occasionally stirred. The maceration filtrate is then filtered. The filtrate obtained was then concentrated with a rotary evaporator at a temperature of 40 °C. Then evaporated in a water bath until a concentrated extract was formed [8].

Phytochemical screening

Phytochemical screening of samples to determine the class of chemical compounds contained in white pomegranate skin includes an examination of alkaloids, flavonoids, tannins, polyphenols, saponins, steroids/terpenoids, quinones, and monoterpenes/sesquiterpenes [8].

Specific gravity test

The clean, dry pycnometer was weighed and then calibrated by setting the weight of the pycnometer and the weight of the water that had been boiled at 25 °C and then weighed (W1). The liquid extract is set at a temperature of approximately 20 °C then put into an empty pycnometer, remove excess extract, adjust the temperature of the filled pycnometer to 25 °C, then weigh (W2) [9].

Intestinal transit method

In this study, white Wistar mice weighing 20-25 grams were used as test animals. Experimental animals were fasted for approximately 18 h, drinking was still given. According to the Federer Formula, the 25 animals were randomly assigned to 5 groups, so each group was 5 animals. Negative control (Na CMC 1 %), positive control (loperamide

HCl 0.0104 mg/20 g mice BW), and test dose 1,2, and 3 (pomegranate peel ethanol extract with a dose of 16, 32, and 64 mg/20 g mice). At time t=0, all groups of test animals were given loperamide HCl or pomegranate peel extract orally according to groups. After t=45 min, the mice were given Carbon-adsorbent orally. At t=65 min, the mice were sacrificed using an anesthetic (ether) and then neck dislocation. Then dissected, the intestines of the mice were carefully removed until they were stretched. The length of the intestine that the carbon-adsorbent marker passed from the pylorus to the tip (which is black) as measured using a ruler. Similarly, the entire intestine length of each animal was calculated as the ratio of the normal distance traveled by the marker to the total gut length [10].

Data analysis

The data were analyzed using the SPSS program, where statistical data tests were carried out first. If the data is normal, ANOVA test is performed to determine the difference between treatments for each group and continued with the LSD (Least Significant Difference) test to determine whether there was a significant difference between treatments for each group. If the data is not normal, then a non-parametric test is carried out [11].

RESULTS

The results of rendemen obtained from simplicia and ethanol extract of white pomegranate peel can be seen in table 1.

Table 1: Rendemen

Simplicia powder	Concentrated extract	% Rendemen
400 g	164.9171 g	41.22 %

Phytochemical analysis of white pomegranate peel (*Punica granatum L.*) was carried out on simplicia and extract. The results of phytochemical analysis obtained from simplicia and ethanol extract of white pomegranate peel can be seen in table 2.

Table 2: Phytochemical analysis

Secondary metabolites	Sample	
	Simplicia	Extract
Alkaloids	-	-
Flavonoids	+	+
Tannins and Poliphenols	+	+
Catechate Tannins	-	-
Gallat Tannins	+	+
Saponins	+	+
Quinones	+	+
Steroids/Triterpenoids	-	-
Monoterpenoids and Sesquiterpenoids	+	+

(+) detected (-) not detected

From the results of the specific gravity, the test obtained an average of 0.9049 g/ml. Specific gravity describes the number of components contained in the substance. The size of the specific gravity value is often associated with the weight fraction of the components contained therein. Therefore, the greater the weight fraction contained in the extract, the greater the specific gravity value [12].

Based on the results of the ethanol-free test on the ethanol extract of the white pomegranate skin (*Punica granatum L.*) with the addition of CH₃COOH (acetic acid) then added with H₂SO₄ (p) (sulfuric acid) and heated. A negative result was obtained (no smell of ester), this indicates that the white pomegranate peel extract is free of ethanol [13]. The results of the intestinal transit test for each treatment group can be seen in table 3.

Table 3: Intestinal transit test

Mice No.	Carbon-adsorbent marker path (cm)				
	Positive control	Negative control	Dose 1	Dose 2	Dose 3
1	0.503	0.763	0.621	0.561	0.464
2	0.509	0.713	0.635	0.521	0.459
3	0.514	0.740	0.659	0.482	0.473
4	0.528	0.754	0.666	0.500	0.492
5	0.540	0.721	0.650	0.537	0.461
mean±SD	0.518±0.015	0.738±0.021	0.646±0.018	0.520±0.030	0.469±0.135

Table 4: Inhibition percentage

Groups	mean±SD	% Inhibition
control (+)	0.518±0.015	29.81 %
Dose 1	0.646±0.018	12.46%
Dose 2	0.520±0.030	29.53%
Dose 3	0.469±0.135	36.44%

Table 5: Test of normality

	Kelompok	Kolmogorov-smimov ^a			Shapiro-wilk		
		Statistic	Df	Sig.	Statistic	Df	Sig.
Rasio	Kontrol positif (+)	.225	5	.200*	.943	5	.687
	Kontrol negatif (-)	.191	5	.200*	.944	5	.691
	Dosis uji I	.183	5	.200*	.958	5	.792
	Dosis uji II	.149	5	.200*	.986	5	.965
	Dosis uji III	.266	5	.200*	.844	5	.175

Based on the results of statistical tests using the IBM SPSS version 26 application for the Kolmogorov-Smirnov normality test, it showed a significance value of 0.200 ($P>0.05$). A significance value of more than 0.05 means that the five variables in the treatment group are normally distributed.

Table 6: Test of homogeneity of variances

Rasio		Levene statistic	Df1	Df2	Sig.
		Based on mean	1.407	4	20
	Based on median	1.025	4	20	.418
	Based on median and with adjusted df	1.025	4	15.929	.424
	Nased on trimmed mean	1.413	4	20	.266

The results of the homogeneity test showed a significance value of 0.268 ($P>0.05$) which means that all the variants were homogeneous. Therefore, the one-way ANOVA test was continued.

Table 7: ANOVA

	Sum of squares	df	Mean square	F	Sig.
Between groups	.244	4	.061	142.014	.000
Within groups	.009	20	.000		
Total	.253	24			

For the results of the ANOVA test where this test aims to determine the mean difference in each group. Based on the results of the ANOVA test showed a significance value of 0.000 ($P<0.05$). A significance value of less than 0.05 indicates that there are significant differences in each treatment group.

Table 8: LSD

Post hoc tests		Multiple comparisons			
Dependent variable: Lsd	Rasio	Mean difference (I-J)	Std. error	Sig.	95% Lower bound
(I) Kelompok	(J) Kelompok				
Kontrol positif (+)	Kontrol negatif (-)	-21940*	.01311	.000	-2467
	Dosis Uji I	-.12740*	.01311	.000	-1547
	Dosis Uji II	-.00200	.01311	.880	-.0293
	Dosis Uji III	.04900*	.01311	.001	.0217
Kontrol negatif (-)	Kontrol positif (+)	.21940*	.01311	.000	.1921
	Dosis Uji I	.09200*	.01311	.000	.0647
	Dosis Uji II	.21740	.01311	.000	.1901
	Dosis Uji III	.26840	.01311	.000	.2411
Dosis Uji I	Kontrol positif (+)	.12740*	.01311	.000	.1001
	Kontrol negatif (-)	-.09200*	.01311	.000	-.1193
	Dosis Uji II	.12540*	.01311	.000	.0981
	Dosis Uji III	.17640*	.01311	.000	.1491
Dosis Uji II	Kontrol positif (+)	.00200	.01311	.880	-.0253
	Kontrol negatif (-)	-.21740*	.01311	.000	-.2447
	Dosis Uji I	-.12540*	.01311	.000	-.1527
	Dosis Uji III	.5100*	.01311	.001	.0273
Dosis Uji III	Kontrol positif (+)	-.04900*	.01311	.001	-.0763
	Kontrol negatif (-)	-.26840*	.01311	.000	-.2957
	Dosis Uji I	-.17640*	.01311	.000	-.2037
	Dosis Uji II	-.05100*	.01311	.001	-.0783

If the results of the ANOVA test are found to be significantly different, further analysis of the LSD (Least Significant Difference) test is carried out. Positive control when compared with negative control, obtained a significance value of 0.000 ($P < 0.05$), indicating that there is a significant difference. The results of the LSD analysis between the negative controls were compared with the test dose I, test dose II and test dose III, a significance value of 0.000 ($P < 0.05$), indicating that there was a significant difference.

Then the positive control was compared with the test dose I, the significance value obtained was 0.000 ($P < 0.05$), indicating that there was a significant difference. However, the positive control compared to the test dose II, the significance value obtained was 0.880 ($P > 0.05$). A significance value of more than 0.05 indicates that there is no significant difference.

The positive control compared with the test dose III, the significance value obtained was 0.001 ($P < 0.05$), indicating that there was a significant difference. The test dose I compared to the test dose II obtained a significance value of 0.000 ($P < 0.05$) indicating that there was a significant difference. The test dose II compared to the test dose III obtained a significance value of 0.001 ($P < 0.05$) indicating that there was a significant difference. The test dose I compared with the test dose III obtained a significance value of 0.000 ($P < 0.05$) indicating that there was a significant difference [14].

DISCUSSION

Based on other research previously, rendement of pomegranate peel ethanol extract was 24.56% [15]. In this study, rendement from the extract was 41.22%; these results indicated that there was an increase in the amount of rendement obtained. The high rendement value indicates the number of bioactive components contained in it [16].

Based on the results of the intestinal transit test, what was observed was the ratio between the length of the intestines of the mice through which the carbon-adsorbent was passed and the length of the intestines as a whole. To measure intestinal peristalsis using a marker, the higher the intestinal peristalsis, the more frequent defecation occurs, which is indicated by the greater the distance traveled by the marker. The principle of this method is to compare the length of the intestine through the marker to the length of the whole intestine. Loperamide is used as a comparison because it can restore cells that are in a hypersecretory state to a normal resorption state and can increase small intestinal transit time and absorption of water, sodium and chloride in the body when electrolyte disturbances occur.

It should be noted that loperamide has a constipating effect by slowing gastrointestinal motility and flow rate from the intestine to the colon and normalizing the balance of absorption and secretion of fluids in the intestinal mucous membrane [17].

The secondary metabolite compounds that have activity in influencing the ratio of Carbon-Absorbent distance are the chelating properties of tannins which have a spasmolytic effect, which can shrink the intestines so that intestinal peristalsis is reduced and can precipitate proteins on the intestinal surface. The astringent properties of tannins will make the small intestine more resistant to chemical stimuli that cause diarrhea [17].

Tannins are classified into two categories, namely hydrolyzed tannins and condensed tannins. Hydrolyzed tannin has a greater astringent ability against diarrhea caused by infection. Tannate protein that is broken down will bind to hydrolyzed tannins that pass through the intestine and reduce secretion from the small intestine, causing constipation [18].

Tannins and flavonoids are useful for treating digestive disorders, one of which is diarrhea because they can inhibit intestinal motility and intestinal fluid accumulation [19].

Punica granatum L. peel extract appears to contain substances that reduce diarrhea by inhibiting intestinal motility and intestinal fluid accumulation. Tannins are known to reduce mucosal secretions and make the intestinal mucosa more resistant. The presence of polyphenolic compounds in *Punica granatum* L. peel extract can

mediate its antidiarrheal properties. The peristaltic inhibitory effect of *Punica granatum* peel extract justifies the use of the plant in traditional medicine and its use as a nonspecific antidiarrheal agent [20]. *Punica granatum* peel extract can inhibit contractions and markedly reduce intestinal motility. This effect may have contributed to the observed antidiarrheal activity. The activity of *Punica granatum* peel extract may contribute to the antidiarrheal effect during infectious diarrhea [21].

Then there are flavonoid compounds with their mechanism of inhibiting the release of acetylcholine which will reduce the activation of nicotinic acetylcholine receptors, which mediate smooth muscle contraction and activation of muscarinic acetylcholine receptors (especially Ach-M3), which regulates gastrointestinal motility and smooth muscle contraction [22]. Flavonoids are well known as antibacterial agents which mechanisms are inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of the attachment and biofilm formation, inhibition of the porin on the cell membrane, and alteration of the membrane permeability, and attenuation of the pathogenicity [23].

Monoterpenoids or essential oils are also able to act as antidiarrheal agents, not only as inhibitors of spasmogenic effects on the intestines but also as inhibitors of growth and development of bacteria that can cause diarrhea such as *Escherichia coli*, *Salmonella*, *Shigella*, *Staphylococcus aureus*, and *Vibrio cholera*. Inhibition of the growth and development of bacteria that cause diarrhea in the intestine will prevent the process of irritation in the intestine and reduce the increase in the speed of intestinal peristaltic movements. This event can reduce intestinal contractions and prolong the absorption time of food in the intestine, thereby stopping the effects of diarrhea [24].

In table 4, regarding the percentage of inhibition, there was a significant difference between the positive controls compared to the test dose group 1. The positive control also has a higher percentage of inhibition value than the test dose group 1, but when compared to the test dose group 2 it has a higher percentage of inhibition value comparable. The percentage of inhibition indicated how effectively the test group compared to control (+) in reducing diarrheal activity. This is calculated by subtracting the value of the carbon path length of the test group by the negative group, then divided by the negative group and multiplied by 100 percent.

Test dose 3 compared with the positive control, test dose 1 and 2 had the greatest percentage of inhibition. Test dose 3 as the largest dose naturally contains more active compounds so that the antidiarrheal activity is most potent. This shows that the ethanolic extract of white pomegranate peel is thought to be able to reduce intestinal motility activity so that intestinal peristalsis was reduced and makes it difficult for carbon-adsorbent to move in the intestine, which makes the distance traveled by carbon-adsorbent becomes shorter [25]. The parameters analyzed statistically was the ratio of the normal distance traveled by the marker to the total gut length.

CONCLUSION

Ethanol Extract of White Pomegranate Peel (*Punica granatum* L.) with dose 1 (16 mg/20 gram mice BW), 2 (32 mg/20 gram mice BW), 3 (64 mg/20 gram mice BW) had antidiarrheal activity as an antibacterial agent, spasmolytic effect, inhibit intestinal motility and intestinal fluid accumulation. The percentage decrease in the ratio of the intestines 12.46%, 29.53% and 36.44%, respectively. The test dose 2 had comparable activity to the positive control and the test dose 3 had highest antidiarrheal activity.

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AUTHORS CONTRIBUTIONS

All the authors contributed equally.

CONFLICT OF INTERESTS

Declared none

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