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Natural Oral Anti-dysentery From Pseudostem Of Klutuk (*Musa balbisiana* Colla) and Kepok (*Musa paradisiaca* L.) Banana Plant From Indonesia

Sri Agung Fitri Kusuma*, Maya Febriyanti, Nisrina Farzadillah
Department of Biology Pharmacy, Faculty of Pharmacy, Padjadjaran University, Sumedang, West Java, Indonesia 45363

Abstract

Aim: The aimed of this study was to investigate the most potent of anti-dysentery candidate from the pseudostem ethanol extract of Klutuk (*Musa balbisiana* Colla) and Kepok (*Musa paradisiaca* L.) banana.

Methods: Each pseudostem of Kepok and Klutuk banana were dried, chopped, and powdered then macerated in 70% ethanol as the solvent. Phytochemical analysis was carried out for both the extracts using standard methods. The anti-dysentery characteristics of both extracts were screened against *Shigella dysenteriae* ATCC 13313 using the agar diffusion method. The Minimum inhibitory concentration (MIC) of the extracts was determined using the macrodillution method by diluting the extract into various concentrations. The minimum bactericidal concentration (MBC) was determined by subculturing the incubation of MIC result on the surface of agar medium using a spread method.

Results: The screening of phytochemical results showed that both pseudostem extracts contain the same metabolites, such as flavonoids, tannins, monoterpenoids, sesquiterpenoids and quinones. Saponin and poliphenol were found only in the pseudostem extract of Klutuk banana. Thus, the pseudostem extract of Klutuk banana has more antibacterial properties than kepok. That was the reason, the anti-dysentery of klutuk pseudostem extract more active than that of kepok. The MIC of the extracts was in the range of 5-10% w/v for kepok, and 2,5-5% w/v for klutuk. The MBC value of the pseudostem extract of kepok was 20-40% w/v and 5%-10% w/v for klutuk.

Conclusion: The pseudostem extract of Klutuk banana more showed the potential as natural oral anti-dysentery compared to kepok.

Keywords: *Musa balbisiana* Colla, *Musa paradisiaca* L., pseudostem, antidysentery, *Shigella dysentriae* ATCC 13313

INTRODUCTION

Bacillary dysentery caused by bacteria of the genus *Shigella*, is one of public health problems in developing countries such as Indonesia. WHO reported that *Shigella dysenteriae* type 1 (Sd1) causes the most severe dysentery disease and is the only strain causative agent for dysentery epidemics in Indonesia. The dysentery cases occur throughout the years, not depend on season [1]. Bacillary dysentery caused by *S. dysenteriae* is transmitted through contaminated food and water by the fecal-oral route, or contact of person-to-person [2,3]. The overcrowd and poor of sanitation hygiene were some factors that improving the risks of dysentery. The common symptoms of bacillary dysentery include diarrhea, abdominal pains, fever, tenesmus and stool with mucus or blood [4]. Every year, 165 million cases of Bacillary dysentery occurred and cause 1.1 million of the deaths [5]. In Indonesia, 29% of toddler deaths were caused by dysentery by Shigella [6]. The delay handling of dysentery infection which lead to dehydration, explained the common reason for the increasing of mortality death caused by Shigella. Therefore, early detection of dysentery and antibiotic therapy were important to be developed.

The determination of effective anti-bacterial therapy is essential for decreasing the prevalence of Shigella. Therapy using antibiotics is very needed for dysentery patients with a high frequency of diarrhea episodes. Moreover, the prolonged diarrhea cause gave significant impact on the poor of nutrition in affected patients. For dysentery patients with bloody stool, the World Health Organization (WHO) recommends for using antibiotics, such as ciprofloxacin, pivmecillinam azithromycin and ceftriaxone [7]. Here, we review the scientific evidence supporting the WHO-recommended antibiotics ciprofloxacin, ceftriaxone and pivmecillinam for the effective treatment of dysentery. In Indonesia, *S. dysenteriae* has been reported to be resistant to several antibiotics, such as: trimethropim, ampicillin, sulfamethoxazole, tetracycline, chloramphenicol and cephalotin [1]. The antimicrobial resistance among Shigella isolates limiting the efficacy of antibiotic treatment. The fact of those resistances, had direct to utilization of medicinal plants and 25–50% of current pharmaceuticals are derived from plant. Crude extracts of the plants possess secondary metabolites which could act as a natural antibiotic for resistance modifying agents [8]. Indonesia is one of the countries that has a high level of plant diversity. For dysentery Indonesian people usually used the banana fruit to stop the diarrhea caused by the effect of dysentery. Our previous research, had found that in certain parts of banana plant variety Klutuk (*Musa balbisiana* Colla) and Kepok (*Musa paradisiaca* L.) banana could be serve as potent anti-dysentery against *S. dysenteriae* 13313 [9, 10]. But, in this study, the other part of the banana plant, that is pseudostem, parts of banana plants that people never use, had been investigated as an anti-dysentery agent. The spread of secondary metabolites can be found in other parts of the same plant. It can even be found an antibacterial secondary metabolite that accumulates higher in the certain part of the medicinal plants. The secondary metabolites which are antibacterial agent were alkaloids, flavonoid, poliphenol, monoterpenoids, sesquiterpenoids and saponin were found the extract of fruit banana. Each of secondary metabolites has a different inhibition mechanism against bacteria,such as: intercalating bacterial DNA, disturbing bacterial membrane integrity, inactivating bacterial adhesin, thus The resultant of their action could be potential for dysentery treatment.

MATERIALS AND METHODS

Materials

The materials used were pseudostem of Klutuk and Kepok banana plants, collected from Cimincrang Village, District Gedebage, Bandung, West Java, Indonesia. The determination of their botanical identities was done in Plant Taxonomy Laboratory of Biology Major, Faculty of Mathematics and Natural Science Padjadjaran University, Indonesia. The identification number of both sample was No. 04/HB/11/2014. *Shigella dysenteriae* ATCC 13313 was used as tested bacteria. The growth media used were *Shigella Salmonella* Agar (SSA-Pronadisa), *Mueller-Hinton Agar* (MHA-Merck), and *Mueller-Hinton Broth* (MHB-Oxoid). The chemicals used were alcohol 96%, ethanol 70%, distilled water, water fuchsine, ammonia, amyl alcohol, disinfectant, chloroform, dimethylsulfoxide (DMSO), acetic acid solution, barium chloride solution, physiological NaCl solution 0.9%, sulfuric acid solution, n-butanol (Bratachem), Lugol's solution, Dragendorf reagents, ferric chloride reagent, Lieberman - Burchard reagent Mayer, technical toluene, carbon dye gentian violet, and vaseline.

Extraction

The pseudostem of each kepok (Musa paradisiaca L.) and klutuk (Musa balbisiana Colla) banana were cleaned using tap water and dried. The dried pseudostem then cut into small pieces and powdered coarsely. The weight of 500 g pseudostem powder were macerated in 3L of 70% technical ethanol. The maceration vessel was closed and allowed to stand for 24 h. The solvent replacement with the same amount was carried out for three times every 24 h. The maceration results were collected and then concentrated with a rotary evaporator to obtain a thick extract. The rendemen of the extracts, then were calculated.

Extract Evaluation

Evaluation of the extracts includes organoleptic observation, water content, phytochemical screening, and confirmation of flavonid using thin layer chromatography (TLC). The organoleptic characters of the extract were carried out based on the senses to describe the shape, color, smell and taste of the extract obtained. Determination of extract water content was carried out by toluene distillation. As much as 2 g of dried extract were put into a clean and dry flask, then 200 mL of toluene were added. The distillation container was carefully heated for 15 min. After all the water was distilled, the receiving tube was allowed to cool to room temperature, then the volume of water was read. The formula of extract water content was calculated by dividing the water volume with the total weight of the extract, then multiplied by 100%. The phytochemical screening was carried out using the standard method which listed on Phytochemical Screening of Plants method [11]. This screening was done to find the group of secondary metabolites in the extract, as follows: alkaloid, flavonoid, poliphenol, tannin, monoterpenoids, sesquiterpenoids, steroid, triterpenoid, quinones, and saponin. Determination of thin layer chromatography profiles (TLC) was carried out to determine the profile of the compounds contained in the extract. Thin Layer Chromatography (TLC) was performed using a thin layer plate. This test was conducted to determine the presence of flavonoid compounds. The stationary phase used was silica gel 60 F254 and the mobile phase was a mixture of chloroform - acetone - formic acid (75: 16.5: 8.5). The Rf value obtained from the spots was detected with UV light 254 nm and 366 nm. This Rf value was obtained by dividing the distance migration of the compound with the distance migration of the developer.

Preparation of Bacterial Cell Suspension

The bacterial cell suspension for anti-dysentery activities test were prepared by adjusting the absorbance of the bacterial suspension to a turbidity standard of 0.5 Mc Farland. McFarland's standard solution consists of two components, $BaCl_2$ 1% solution and 1% H_2SO_4. A total of 0.05 mL of 1% $BaCl_2$ solution was mixed with 9.95 mL of 1% H_2SO_4 solution and shaken until homogeneous. Turbidity of the solution was measured at a wavelength of 620 nm by using distilled water as a blank. The absorptive value of the standard solution must be in the range of 0.08 to 0.13. McFarland 0.5 standard solution is equivalent to bacterial cell suspension with a concentration of 1 x 10^8 CFU / mL. A loop full of *S. dysenteriae* colonies was aseptically inoculated into a 10 ml of sterile normal saline (0.9%). The turbidity of each bacterial suspension was adjusted to equal that of 10^8cfu/ml [9].

Anti-dysentery Test Activity

Anti-dysentery test activity of each extract was employed using the agar diffusion method with perforator technique. The concentration variation used for both extracts was different, based on previous orientation results. The start concentration of pseudostem extract of kepok banana (40, 50 and 60% w/v) was higher than that of Klutuk banana ((20, 30, 40,50 and 60% w/v). Each concentration of the extracts was diluted using DMSO. A volume of 20 µL 0.5 McFarland *S. dysenteriae* suspensions were poured into a sterile petri dish, then a volume of 20 ml MHA was added into the petri dish. The petri dish was homogenized slowly, then allowed to solidify. The solid medium was perforated aseptically and the formed holes were used to store a volume of 100 µl extract, then incubated at 37 °C for 24 h. The inhibition zone diameters of each concentration were measured using calipers.

Statistical Analysis

The statistical analysis was conducted to study the effect of extract concentration to the inhibitory diameter. The concentrations used in anti-dysentery activity and the resulted diameters were processed using SPSS 21 software. The diameter for each extract was analyzed using one-way analysis of variance (ANOVA). P value < 0.05 was considered as significant. If the analysis results were significantly different at the 5% test level ($\alpha \leq 0.05$), then the Tukey test was done [12]. In addition, to determine the effect of different extract, the students-T test was used [13].

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

The tests were aimed to determine the MIC value of the test extract which can inhibit the 90% microbial growth of the initial colonies number and the MBC value that can kill 99.9% of bacterial colonies. The MIC values of both extracts were determined by diluting the extract into various concentrations (40%, 20%, 10%, 5%, 2.5%, 1.25%, 0.625%, 0.3125%, 0.15625% and 0.078125% w/v) using the microdilution method. In the each well of the 96 wells-microtitreplate, a volume of 100 µl MHB was poured as diluting agent. The first well was used as a negative control contained only MHB media and the 12^{th} well as a positive control contain of MHB media and bacterial suspension. The ethanol extracts of pseudostem extract in a concentration 0f 80 %w/v were dissolved using DMSO, followed by serially twofold dilution using MHB medium to achieve the lower concentration. To the second well, a volume of 100µl extract was added and homogenize, then pipetted a volume of 100µl from the second well to the 3rd well. This treatment was repeated and carried out in a row until the 11^{th} well. Furthermore, a volume of 100µl bacterial suspension with a concentration of 10^6 CFU/ml was inoculated to each well, thus the final inoculum in each well was $5x10^4$CFU / 200µl. The test medium was incubated at 37 °C for 18-24 h. The MIC result which did not show bacterial growth (clear), were dropped on the surface of agar medium and then incubated at 37 ° C for 18-24 h. The growth of colony was counted manually. Based on the MBC calculation, the amount of the 0.01% colonies number was 0.25 colonies or in the other words, there should be no colonies at all.

RESULTS AND DISCUSSION

Extraction Results and Evaluation

The quality of both extracts was examined for the extract organoleptic, rendement extract, water content, phytochemical screening, and confirmation of flavonid using thin layer chromatography (TLC). Based on the results of organoleptic observation, both ethanol extracts of banana pseudostem exhibited the same extract morphology in the form of thick liquid, typical extract and bitter odor. The difference between the two extracts was the color of the extract, where the extract of kepok extract was greenish brown, while the Klutuk banana had dark brown in color. The extraction of 500 g kepok banana pseudostem yielded 19.195 g extract and the rendemen was 3.839%. Meanwhile the klutuk banana pseudostem yielded 22.35 g and the rendement was 4.47%. The water content of the Klutuk pseudostem extract was 0%, lower than that of kepok, which was 5%. The results were

complied the requirement of water content in the extract, should not exceed 10% [14].

The screening of phytochemical results showed that both pseudostem extracts contain the same metabolites, such as flavonoids, tannins, monoterpenoids, sesquiterpenoids and quinones. Saponin and poliphenol were found only in the pseudostem extract of Klutuk banana. Thus, the pseudostem extract of Klutuk banana has more antibacterial properties than kepok. Actually, flavonoid, tannins, saponins, poliphenols, monoterpenoids, sesquiterpenoids and quinones, were obtained in other parts of kepok and klutuk banana plants [9,10]. Even though the secondary metabolites are not primarily essential for the metabolism of the plant, but they can protect the plants from microbes or herbivores by interfering the molecular target [15, 16]. Flavonoid is a wide spectrum of antimicrobial with the inhibition mechanism by increasing the permeability of the membrane and interacts with the protein membranes present in bacterial cell wall [17]. The antibacterial mechanism of tannins are inactivation of cell envelope transport protein and microbial adhesin [18]. The terpene derivatives are also reported has natural antibacterial by disrupting the bacterial membrane [19]. For, quinones, the inhibition targets are adhesins protein which is exposed to the surface, polypentides of cell wall, and membrane-bound enzymes [20]. Saponin content in the pseudostem extract from klutuk bananas can improve its antibacterial effect, because it can work to damage the cell membrane.

Determination of thin layer chromatography profiles (TLC) was carried out to determine the profile of the compounds contained in the extract. Based on the experiment, a solvent phase (chloroform - acetone - formic acid= 75: 16.5: 8.5) was used to dissolve and assist the compounds in the extract to migrate along the silica plate with capillary action. From the resulting spots appeared on the TLC plate associated with solvent system A, we can deduce that 70:30 mixture of hexane: acetone solvent has a low eluting power compared with solvent system B. This is because th e chemical compound insolvent system A ascended much slower than a solvent system B in a given time. As we known, the TLC plate is made up of silica gel, a very polar adsorbent which is likely to absorb the polar compound strongly. The TLC result was performed in figure 1 and table 1-2.

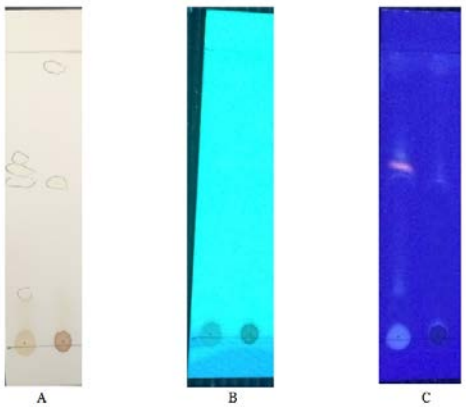

Figure 1: Resulting spots on TLC plate

Notes: visible light (A); 254 nm of UV light (B); 366 nm of UV light (B); pseudostem extract of Kepok (1); pseudostem extract of Klutuk (2)

From the spots exhibited on the TLC plate, pseudostem extract of kepok had 4 nonpolar compounds, while the klutuk pseudostem ethanol extract had 2 non-polar compounds. The spots were light yellow and yellow or orange fluoroside. The type of flavonoid that might be was flavonols containing free 3-OH and or not having 5-OH free [21]. In the pseudostem ethanol extract of kepok, there was a red spot which indicated the presence of chlorophyll compounds. Chlorophyll compounds cannot dissolve in water but dissolve in ethanol, methanol, chloroform and acetone.

Table 1: TLC Result of Kepok Banana Pseudostem Etanolic Extract

Spot No.	Rf	Visible	Ultraviolet (nm)	
			254	366
1	0.15	-	-	greenish yellow
2	0.525	-	-	greenish yellow
3	0.5625	Light yellow	-	Orange fluososide
4	0.6125	-	-	greenish yellow

Table 2: TLC Result of Klutuk Banana Pseudostem Etanolic Extract

Spot No.	Rf	Visible	Ultraviolet (nm)	
			254	366
1	0.525	Light yellow	-	red
2	0.9	-	-	yellow

Antibacterial Activity Results

The antibacterial activity results of both pseudostem extract exhibited good activity *S. dysenteriae* at different concentrations. The pseudostem extract of Klutuk showed a higher diameter of inhibition than that of Kepok. This antibacterial data correlated with the phytochemical screening results that pseuodostem of Klutuk contain more antibacterial compounds than Kepok. The mean inhibitory zone of both ethanol extracts against *S. dysenteriae* was summarized in table 3. To determine the effect of increasing extract concentration on the inhibitory zone formed, statistical analysis was carried out. The analytical method used was one way analysis of variance (ANAVA) and continued with the Tukey test. The Tukey test was conducted to determine the difference in the ability of each extract concentration to the inhibition zone diameter.

Table 3: Diameter of Antibacterial Activity

Extract concentration (%w/v)	Inhibitory diameter zones (mm)	
	Kepok	Klutuk
20	-	11.7 ± 0.000
30	-	12.8 ± 0.000
40	9.15± 0.000	13.1± 0.000
50	10.1± 0.000	13.4± 0.000
60	10.6± 0.000	13.6± 0.000
Positive control	+	+
Negative control	-	-

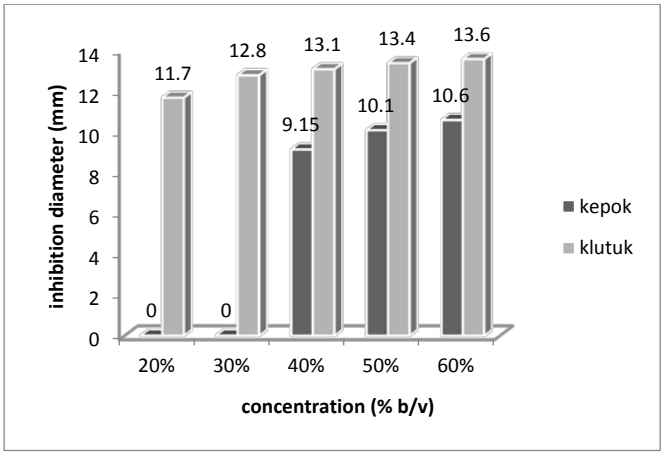

Figure 2: Comparatison of inhibition anti-dysenteriae activities of Klutuk and Kepok pseudostem extract

Table 4: Anova Test Result of Kepok Pseudostem Antidysentrial Activity

Source	Type III Sum Of Squares	Df	Mean Square	F	Sig.
Corrected Model	2.651	2	1.325	58.901	0.000
Intercept	913.047	1	913.047	40579.86	0.000
Concentration	2.651	2	1.325	58.901	0.000
Error	0.135	6	0.023		
Total	915.833	9			
Corrected Total	2.786	8			

Table 5: Anova Test Result of Klutuk Pseudostem Antidysentrial Activity

Source	Type III Sum Of Squares	Df	Mean Square	F	Sig.
Corrected Model	5.791	4	1.448	76.433	0.000
Intercept	2524.87	1	2524.87	133308.9	0.000
Concentration	5.791	4	1.448	76.433	0.000
Error	0.189	10	0. 019		
Total	2530.85	15			
Corrected Total	5.98	14			

Table 6: Tukey Test Result of Kepok Peel Antidysentrial Activity

Concentration (% w/v)	N	Subset	
		1	2
40	3	9.3167	
50	3		10.3333
60	3		10.5667
Sig.		1.000	0.217

Table 7: Tukey Test Result of Klutuk Peel Antidysentrial Activity

Concentration (% w/v)	N	Subset			
		1	2	3	4
20	3	11.8667			
30	3		12.8		
40	3		13.1667	13.1667	
50	3			13.37	13.37
60					13.6667
Sig.		1.000	0.052	0.419	0.135

From the statistical result on table 4, it can be seen that with the real level $\alpha = 0.05$, the value of sig = 0 or smaller than the real level, made the initial hypothesis was rejected. Thus, there was an effect of increasing the concentration of pseudostem ethanol extracts from kepok banana on the inhibitory zone formed. Similarly, the statistical results on Klutuk pseudostem extract which were listed in table 5. The results of the Tukey test (table 6) showed that the pseudostem extract of kepok banana with concentration of 40% (b/v) has a significant difference in inhibitory zones with concentrations of 50% (b/v) and 60% (b/v). While the concentrations of 50% (b/v) and 60% (b/v) did not have a real difference in inhibitory zones because they were in the same column. Whereas the results of the Tukey test for pseudostem Klutuk, with a concentration of 20% (b/v) had a significant inhibition zone difference with other concentrations of 30%, 40%, 50% and 60% (b / v). Whereas for concentrations of 30% (b/v) did not have a significant difference in inhibitory zones with a concentration of 40% (b / v) but had significant differences with other concentrations. Likewise with concentrations between 40% - 50% (b / v) and 50% - 60% (b / v) which did not show significant differences in the inhibition zones formed, but have significant differences with other concentrations. Then to compare the difference between the pseudostem extract of Klutuk and kepok banana, T test was done by comparing the two results of the extract activity test. For the results of the T test can be seen in table 8.

Table 8: T-Test Result of Pseudostem Extract Between Klutuk and Kepok Banana Plant

		T-test for Equality of Means		
		Sig. (2-tailed)	Mean Difference	Std. Error Difference
Potency	Equal variances assumed	0	-2.90178	0.26614

From the results of the T test, it was obtained that the value of sig = 0 or smaller than the real level of 0.05. This showed that the initial hypothesis was rejected, which means that there was a difference between the results of the activity test of kepok and klutuk pseudostem ethanol extract.

MIC and MBC Determination

In determining the minimum inhibitory concentration (MIC), the microdilution method was used by the turbidimetry method through observing the turbidity which indicated the growth of test bacteria. Whereas in determining the minimum bactericidal concentration (MBC), bacterial subcultures from MIC result were carried out by taking and inoculating the bacterial suspension on the surface of the MHA agar medium. The purpose of determining the MIC value was to find out the lowest concentration of klutuk and kepok banana pseudostem ethanol extract which could still inhibit the bacterial growth, meanwhile determining the KBM value was done to determine the lowest concentration of klutuk banana and kepok banana pseudostem ethanol extract which could kill 99.9% of the *S. dysenteriae* [22]. The MIC value was shown in the lowest concentration solution that looked clear on microtiter plates. KBM value was found in the agar medium with the lowest concentration of solution that had no bacterial growth. The results of the MIC determination of kepok and klutuk pseudostem ethanol extract were presented in table 9.

Table 9: Pseudostem Extract MIC Results

Concentration (% w/v)	Pseudostem Extract	
	Kepok	Klutuk
40	-	-
20	-	-
10	+	-
5	+	-
2.5	+	+
1.25	+	+
0.625	+	+
0.3125	+	+
0.15625	+	+
0.078125	+	+

Notes : (-) bacterial growth was absence; (+) bacterial growth was presence

From the results, the MIC value of Kepok pseudostem (10 <MIC<20%w/v) was higher than that of Klutuk(2.5 <MIC<5%w/v). It mean that the pseudostem extract of Klutuk more potent as an anti-dysentery than Kepok. For the MBC value, an ethanol extract of kepok pseudostem with no colony growth was 40% (w/v) and at a concentration of 20% (w/v), there were 34 colony growth. Whereas the concentration of klutuk pseudostem ethanol extract with no colony growth was 10% (w/ v) and at a concentration of 5% (w/ v), there were 25 colony growth. Based on calculations, only 0.25 bacterial colonies can still grow as the range of KBM ranges. So that the MBC value of the kepok

pseudostem ethanol extract was at concentrations between 20-40% (w/ v) and for klutuk was between 5-10% (w / v).

Table 10: Pseudostem Extract MBC Results

Concentration (% w/v)	Pseudostem Extract	
	Kepok	Klutuk
40	-	-
20	34 colonies	-
10	+	-
5	+	25 colonies
2,5	+	+

Notes : (-) bacterial growth was absence; (+) bacterial growth was presence

CONCLUSION

Our results demonstrated that the pseudostem ethanolic extract of the Klutuk banana plant could be a novel candidate for natural dysentery treatment.

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