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Synergistic Combination Effect of Klutuk Banana Fruit Extract (*Musa balbisiana* Colla) With The Pseudostem Extract As Natural Anti-dysentery And Anti-hypokalemia Candidate

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Abstract

Aim: This study was aimed to demonstrate the synergistic combination effect of klutuk banana fruit extract (*Musa balbisiana* Colla) with the pseudostem extract as natural anti-dysentery and anti-hypokalemia candidate.
Methods: The anti-dysentery and synergistic properties of the extract combinations were assayed using the agar diffusion method with perforation techniques. Followed by potassium content determination of extract combination using Atomic Absorption Spectrophotometry.
Results: The results showed that the combined effect of the fruit and pseudostem extract led to a synergistic effect, both as anti-dysentery and anti-hypokalemia candidate. From this study, the best extract combination as anti-dysentery and anti hypokalemia was a 0.6 g of fruit extract combined with 1.6 g of pseudostem extract. This combination resulted in an inhibition diameter of 21.4 mm that categorized as a strong antibacterial agent and achieved potassium levels at 0.1493 %, close to 1.5% potassium content in the standard oral rehydration solution.
Conclusion: Therefore, the study revealed that the combination of fruit and pseudostem extract of Klutuk banana can be used as a potent and novel anti-dysentery and anti-hypokalemia agent.

Keywords: klutuk, Musa balbisiana colla, fruit, pseudostem, anti-dysentery, hypokalemia

INTRODUCTION

The dysentery is an intestinal disease that primarily occurs in the colon and can lead to severe diarrhea with mucus and blood mixed in feces. In the area tropical and subtropical, the main cause of dysentery is *Shigella dysenteriae* with a death rate of 6.2% [1]. In Indonesia, 29% of deaths caused by bacillary dysentery occurred at age 1-4 years old [2]. Shigella has the ability to invade intestinal epithelial cells and release toxins that can cause irritation and intestinal injury. Its enterotoxins can cause diarrhea and blood. Moreover, the most often complications due to Shigella is dehydration [3,4]. Patient with high frequency in diarrhea or vomiting, should consume more fluids to prevent dehydration. The diarrhea can disturb the body fluid and electrolyte, especially lowering the potassium levels of body causing hypokalemia. Moreover, from others study reported that hypokalemia is associated with hypertension patients, acute myocardial infarction, heart failure and diuretic patients [5-10]. Therefore, it is very important to prevent hypokalemia and to maintain plasma potassium levels in the upper normal range. In addition, dysentery sufferers often do not heed the effects of dehydration that occur due to persistent diarrhea. For that, in the future, a treatment strategy that can be given at once in the form of antibiotics and anti-hypokalemia is needed.

The increasing antibiotic resistance to ampicillin, chloramphenicol, tetracycline, co-trimoxazole, nalidixic acid, gentamicin, and 1st-2nd generation cephalosporin make those antibiotics less effective for dysentery treatment [11]. Therefore, it must be emphasized that dysentery patient needs new antibiotic development for the widespread Shigella strain. This leads the scientist to investigate a new antibiotic candidate from herbal to treat dysentery. The point was supported by WHO to use traditional medicines in diarrheal disease, combined with education to improve and prevent diarrhea [12]. The use of traditional medicines has been proven to be used as a natural antibiotic, especially in bacillary anti-dysentery. Based on previous studies, the ethanolic extract of Klutuk banana fruit performed the strongest anti-dysentery effect among other parts of klutuk and kepok banana variants. But, even though of the pseudostem parts of Klutuk banana plants had low anti-dysentery potency, it produced the highest potassium level among all parts of Klutuk and Kepok banana variants. Thus, the combination of klutuk banana fruit extract and its pseudostem extract could improve the synergistic effect to treat dysentery and to prevent hypokalemia.

MATERIALS AND METHODS

Plant Materials

The herbs used in this study were unripe fruit and pseudostem part of the Klutuk banana with age of 3 months and collected from Cimincrang Village, Gedebage District, Bandung, West Java, Indonesia. The identification of those herbal was determined in Plant Taxonomy Laboratory of Biology Major, Faculty of Mathematics and Natural Science Padjadjaran University. The specimen number of the klutuk banana plant was HB/04/2016.

Chemical materials

The chemicals used consist of ethanol (CV. Agung Menara Abadi), aquadest (Chemistry dept. UNPAD), hydrochloric acid (Merck), acetic acid (Merck), amyl alcohol (Merck), sulfuric acid (Merck), dimethyl sulfoxide (Merck), physiological NaCl (PT. Widrata Bakti), chloroform (Merck), ether (Merck), methanol (Merck), mayer reagents (Merck), dragendorff reagents (Merck), iron (III) chloride (Merck), 1% gelatin solution (CV. Medilabs), metal powder (CV. Agung Menara Abadi), ammonia solution (Merck), Liebermann Burchard reagents (Merck), vanillin (merck) and sodium hydroxide (PT. Brataco). The growth medium used in this study were Salmonella Shigella agar (Pronadisa), Mueller Hilton Agar (Oxoid) and Mueller Hilton Broth (Oxoid).

Shigella Strain

Shigella dysenteriae ATCC 13313 was obtained from the Laboratory of Microbiology, Faculty of Medicine, University Maranatha Kristen, Bandung.

Extraction

The fruits and pseudostem of klutuk banana were cleaned using aquadest, dried, and chopped. Then the slices of materials were dried at room temperature to produce simplicia with water content comply the standard simplicia.The weight of each simplicia was 500 g for the fruit and 1 kg for the pseudostem. Each of the material was soaked in 70% ethanol as much as 3 L and 5 L, for the fruit and peseustem simplicia respectively. The maceration

process was allowed for 24 h with filtrate taken and solvent replacement every 24 h. Then all macerates of each extract were evaporated to achieve a thick extract.

Extract Parameters Evaluation

Organoleptic examination was used sensory testing, including: the shape, color, smell, and taste of the extract. Extract yield was calculated by dividing extract weight with simplicia weight and then multiplied by 100%. Determination of extract water content was carried out by toluene distillation. For the water content calculation, the 2 g of each extract was put into distillation container and added by 200 ml of toluene, followed by heating for 14 min. After all the water was distilled, the receiving tube was allowed to cool to achieve the room temperature. Then the water volume was observed. The water content value was calculated by dividing the water volume with the extract weight and multiplied by 100%.

Phytochemical Screening and Thin Layer Chromatography (TLC)

Phytochemical screenings of each extract were carried out to determine the secondary metabolite content, such as alkaloids, flavonoids, tannins, Quinones, polyphenolates, monoterpenoids, sesquiterpenoids, triterpenoids, steroids, and saponins. The screening method was performed according to standard methods [13]. The TLC of each extract was using thin layer plates with silica gel 60 F254 as the stationary phase and a mixture of chloroform - acetone - formic acid (75: 16.5: 8.5) as the mobile phase. Rf values were obtained from the spots which detected using UV light at 254 nm and 366 nm. The Rf value was obtained by dividing the compound distance migration with the migration distance of the developer.

Determination of Potassium Concentration in Each Extract

The potassium content in each extract was determined before both extracts were combined, to predict the calculation of potassium levels in accordance with the ORS in the market. The potassium content test was done by preparing the sample using the dry destruction method, then the samples were analyzed using Atomic Absorption Spectrophotometry (AAS). The ethanol extracts of klutuk banana fruit and pseudostem were weighed each of 1 g, then heated in the furnace at a temperature of 600^0C to form ash. A volume of 1.5 ml 6.5% HNO_3 was added to the ash and reheated on a hot plate and then filtered into a 10 ml volumetric flask. Demineralized water was added to the tare limit of the flask, then the sample was ready to be measured [14]. Sample concentration analysis was carried out by making potassium standard solution, making calibration curves, and analyzing the potassium content of samples. Before dissolving, solid KCl was weighed for 1 g and dried at 100^0C for 2 h then cooled for 30 min. After that, a 25 mg KCl was weighed and put into a 25 ml volumetric flask, then demineralized water was added to obtain a 1000 ppm stock solution. The 1000 ppm of stock solution was diluted to obtain concentrations of 100, 200, 300, 400, and 500 ppm. Each absorbance solution was measured with demineralized water as blank using Atomic Absorption Spectrophotometry (AAS). The measurements were carried out from the smallest concentration to highest concentration. The measurement results were recorded and calculates as a linear regression equation: y =ax + b. The R value was calculated to determine the linearity.

Preparation of 0.5 Mc Farland Bacterial Suspension

The Mc Farland standard solution was prepared by mixing 0.05 mL of $BaCl_2$ 1.175% solution with 9.95 mL of 1% H_2SO_4 solution, then shaking it until it was homogeneous. The solution turbidity was measured at 625 nm using distilled water as the blank. The absorbance value of the solution must be in the range of 0.08 to 0.13 to achieve the absorbance of 0.5 Mc Farland solution. The McFarland 0.5 standard solution is equivalent to bacterial cell suspension, which has a concentration of 1 x 10^8 CFU/ mL [15]. The bacteria colonies which had been cultured in SS agar medium for 18-24 h at 37 ° C were taken one Ose, then suspended in physiological NaCl sterile. Bacterial turbidity was measured with McFarland 0.5 standard solutions.

Combination Effect

This combination effect test was previously done using fixed concentration to determine the antibacterial effect of both extracts in combination. If the result produced synergistic effect, then the antibacterial test using 4 different concentrations would be continued. A volume of 20 µl bacterial suspension was poured into a sterile petri dish, then suspended homogenously by a 20 mL of MHA medium (40-45^{0}C) and allowed it to solidify. Then, the medium was perforated in adjacent positions for two holes and in opposite positions for the other three holes. In two adjacent holes, each hole was filled with an extract with a concentration of 100% b/v as much as 50 µl. For the other two holes, each hole was filled by each extract with a volume of 50 µl at the same concentration. In the other hole, 50 µl of each test extract were put in one hole, so that the total extract included was 100 µl.

Combination Design of Extract Concentration

The extract concentration used in combination was adjusted based on the content of potassium in the commercial anti-dehydration on the market, which was 1.5 g / L or 0.15 g / 100 ml. The content of potassium in the combination of extracts used must be equivalent to the level of potassium. It is known from our previous study that 1 g klutuk banana ethanolic extract contains 0.041 g potassium. Thus, to achieve the potassium level comply to potassium concentration in the oral electrolyte, 0.109 g of potassium was needed from the ethanol extract of klutuk banana pseudostem. For extract concentration of 80% b/v (0.8 g), fruit ethanol extract containing 0.033 g of potassium combined with 1.5 g pseudostem extract that contain 1.467 g of potassium. The same calculation for concentrations of 20, 40, and 60% was done to achieve the final potassium level as much as commercial oral rehydration solution. The final extract combination could be seen in table 1.

Extract Combination

Combination	Fruit Extract (g/mL)	Pseudostem extract (g)
1	0.2	1.8
2	0.4	1.7
3	0.6	1.6
4	0.8	1.5

Antibacterial Activity of Extract Combination

Antibacterial activities of extracts combination were carried out using the agar diffusion method with perforation technique. The ethanol extracts of fruit and pseudostem Klutuk banana were weighed according to the combination design, then dissolved in 1 ml of DMSO. A volume of 20 µl bacterial suspension and 20 ml sterile MHA agar (temperature 40-45^{0}C) were aseptically poured into sterile petri dishes. Then, the media was homogenized and allowed to solidify. After that, the test media were perforated and the holes were filled with each combination of 100 µl extracts. In this step, a positive control, negative control, and DMSO control were made as confirmation of the test results. The positive control contained 5 mL MHA and 5 µl bacterial suspension, while negative controls contained only MHA media. The DMSO control contains MHA, DMSO, and test bacteria. All test media were incubated for 18-24 h at 37 °C.

Statistical Analysis

The antibacterial activities results of the extracts combination were analyzed using SPSS 17 software with one way variance analysis (ANAVA) method to determine the effect of the concentration of extract combinations on the inhibitory zone diameter [16]. If the results got significant effect, then it would be proceeding with the Tukey test to determine whether there were

differences in the ability of each concentration of combinations in inhibiting bacterial growth [17]. T-test was then carried out to compare the combined activity of ethanol extract of klutuk banana fruit and pseudostem with the ethanol extract of klutuk banana fruit as single extract [18].

Potassium Level Determination of Extracts Combination

The potassium level of extracts combination in variation ratio was implemented using the same procedure as the single extract. The determination of potassium level was conducted using AAS.

Results And Discussion

Yield of Extraction

A total yield extract from the fruit and pseudostem were respectively 35.5 g and 37.1 g. The rendemen percentage of both extract was 7.1% of the fruit extract and 3.71% from the pseudostem extract. The extract yield value is related to the number of extracted secondary metabolites [19]. In the maceration process, the solvent will penetrate the cell wall of simplicia then enter the cell cavity containing the active compound. Ethanol is a solvent that can increase the permeability of cell walls, so that both polar and non-polar substances can be pulled out. Thus, the active compound will dissolve in the extracting solution. There is a difference in the concentration of active compounds that are outside the cell and inside the cell so that the concentrated solution will be pushed out of the cell [20].

Extract Characteristics

The extracts were characterized for its morphological appearance and water content. The ethanolic extract of fruit klutuk banana and pseudostem showed the same characteristic in morphology and taste. The characteristics of both extracts were dark brown, bitter taste, and distinctive odor. Determination of the water content was carried out to determine the percent of water content contained in the extract. From the results, The water content of both extracts was 5%. This water content value has complied with the standard of extract water content that is not more than 10% [21].

Phytochemical Screening Results

The phytochemical screening analysis revealed the presence of flavonoids, polyphenols, tannins, monoterpenoids and sesquiterpenoids, quinones, and saponin in both of the ethanol extract of fruit and pseudostem klutuk banana. Secondary metabolites are non nutritive phytochemicals and properties that can protect or prevent disease. Characteristic of fruit banana was in immature stages. The reason for this, where the empirical use as a traditional herb for anti diarrheal and based on other research which reported that there was a difference in phenol content between banana stage of development [22]. The fruits at immature stages display higher phenol contents than at the ripe stages [23,24]. The polyphenols can cause bacterial cell membrane lysis by coagulating the bacterial protein. Flavonoids can form complex compounds with protein extracellular and can damage the bacterial cell membrane followed by discharge intracellular compounds [25]. Meanwhile, tannins can lead to bacterial lysis through cell membrane damage and cell wall shrinkage. Flavonoids and tannins have been studied to exhibit medicinal activity [26]. The saponins also plays a role as an antibacterial agent by interacting with lipopolysaccharide of bacterial cell wall thus saponin can increase the cell wall and reduce the surface stress [27]. The phytochemicals use of fruit and pseudostem banana extract with known antibacterial properties, can be harnessed in dysentery treatment.

Thin Layer Chromatography Results

Determination of Thin Layer Chromatography (TLC) profile was carried out to determine the compound profile of fruit and pseudostem ethanol extract. The silica as the stationary phase can generally separate amino acid compounds, phenols, alkaloids, fatty acids, sterols, and terpenoids. The mobile phase used was non polar. This mobile phase is a specific for the separation of flavonoid compounds. The TLC results of both extracts were displayed in figure 1 and table 1-2.

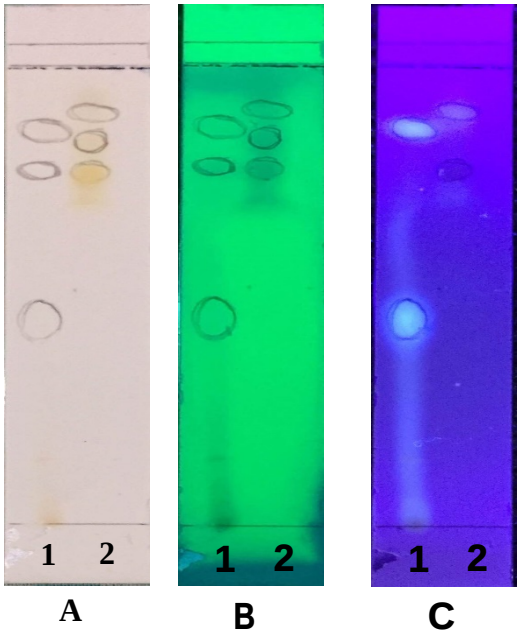

Figure 1: (a) Thin layer chromatography (TLC) of extract in visible light. (b) TLC of extract in UV- wavelength of 254 nm (c) TLC of extract in UV-wavelength of 366 nm; (1) fruit ethanolic extract of Klutuk banana; (2) pseudostem ethanolic extract of Klutuk banana

Table 1: TLC Profile of Fruit Klutuk Banana Ethanol Extract

Spot No.	Rf	Visible light	UV light	
			254 nm	366 nm
1	0.447	-	-	greenish yellow
2	0.776	-	blue	-
3	0.868	-	-	greenish yellow

Table 2: TLC Profile of Pseudostem Klutuk Banana Ethanol Extract

Spot No.	Rf	Visible light	UV light	
			254 nm	366 nm
1	0.776	yellow	blue	Dark blue
2	0.842	Ligth yellow	blue	-
3	0.895	-	-	greenish yellow

Each extract has three spots detected in UV light of 254 and 366 nm. The profile TLC of both extracts performed the presence of three non-polar compounds. Based on the literature, flavonoid group compounds will produce yellow, green, or blue fluorescence depending on the type of structure [28]. Types of flavonoids that produce yellow or orange fluorescence and blue fluorescence are thought to be flavonols which have free 3-OH or no free 5-OH, flavones, and flavanones which do not contain 5-OH [29].

Potassium Concentration in Each Extract

Dry destruction was carried out at temperatures above 500^0C where at this temperature the sample will be completely deformed and without inorganic components evaporation. The function of

HNO_3 as a strong oxidizing agent is to break the bonds of complex organometallic compounds. The addition of HNO_3 is followed by heating to accelerate the breaking of the organometallic bond into inorganic compounds [30]. The absorbance of potassium standard solution with a concentration of 100, 200, 300, 400, and 500 ppm were measured to obtain the following linear regression equation: y = 0.00043 + 0.0000246 x with a correlation coefficient of 0.999130. From the results, it was found that the potassium concentration of fruit and pseudostem klutuk banana ethanol extracts were 4884 ppm (4.169%) and 8328 ppm (7.656%) respectively.The potassium content was higher produced by pseudostem than from its fruit of Klutuk banana. Thus, the combination of both extracts could provide double pharmacology effect as a traditional medicine that potential as antidysentery and potassium deficiency.

Combination Effect

The combination effect test was aimed to determine the bacterial inhibition efficacy of both extract if its combined. The synergistic effect was observed when the effect of the combined extract was greater than the sum of the individual extract effects. The combination extract was determined to have a synergistic effect if the anti-dysentery activity was getting bigger. In contrast, it was said as antagonistic if the activity gets smaller. In this study, the synergistic effect of both extracts against *S. dysenteriae* was resulted when the fruit extract combined with pseudostem extract. According to these results, the combination of fruit extract combined with pseudostem extract showed a synergistic effect against *S. dysenteriae* ATCC 13313. The inhibition zones of extract combination were bigger than alone. These results can be seen in table 3. Both combinations of extract in a certain concentration showed a significant anti-dysentery characters against *S. dysenteriae* ATCC 13313. Considering the increasing rate of *S. dysenteriae* resistance, it is very important to control the spread of dysentery disease. Because the synergism between the fruit and pseudostem extract of Klutuk banana could provide a double inhibition for dysentery treatment due to sensitive and resistant *S. dysenteriae*.

Table 3: Anti-dysentery Combination Effect Of Fruit and Pseudostem Extract

Extract (100% b/v)	Diameter of Inbitory Zone (mm)
Fruit	18.85 ± 0.05
Pseudostem	17.05 ± 0.05
Combination 1	21.20 ± 0.00

Note: perforator diameter : 9 mm

Those results were in accordance with our previous studies which showed that the ethanol extract of banana fruit was more potent as an anti-dysentery compared to pseudostem extract. But, in this combination, the pseudostem extract was targeted as potassium supplier due to it had the high potassium content among all parts of the banana Klutuk plant extract. Therefore, in this study, there were four design combination of the extracts which were adjusted based on the content of potassium in the commercial anti-dehydration on the market, which was 1.5 g / L or 0.15 g / 100 ml. The results were summarized in table 4-5.

Table 4: The Diameter of Fruit Extract Inhibitory Zone

Extract concentration (% b/v)	Diameter of Inbitory Zone (mm)
20	13.00 ± 0.05
40	15.20 ± 0.05
60	16.20 ± 0.00
80	17.10 ± 0.05

Note: perforator diameter: 9 mm

Table 5: Inhibition of Extract Combination Design

No.	Design of Extract Combination		Diameter of Inhibition (mm)	Antibacterial Categories [31]
	Fruit Extract (g)	Pseudostem Extract (g)		
1	0.2	1.8	19.7 ± 0.05	moderate
2	0.4	1.7	21.2 ± 0.05	strong
3	0.6	1.6	21.4 ± 0.05	strong
4	0.8	1.5	21.6 ± 0.05	strong
5	DMSO	-	0.00± 0.00	No activity

Note: perforator diameter : 9 mm

The extract combination design results revealed that the addition of pseudostem extract in the fruit extract showed a great synergistic effect with a significant increase in inhibition diameter against *S. dysenteriae* in the increasing of fruit extract concentration. In first combination design, the antibacterial activity increased from 13.00 ± 0.05 mm for fruit extract alone to to 19.7 ± 0.05 mm for the combination. The inhibition zones were displayed in figure 2. Inhibition diameter enlargement over 5 mm was considered significantly as strong antibacterial. The increasing diameters were similar to those of resulted by others combination extracts design. Based on the Clinical and Laboratory Standards Institute (CLSI), there are three categories of antibacterial according to the diameter of the inhibitory produced, as follows: strong antibacterial (≥ 20 mm); moderate antibacterial (15-19 mm); and weak antibacterial (≤ 14mm) [31].

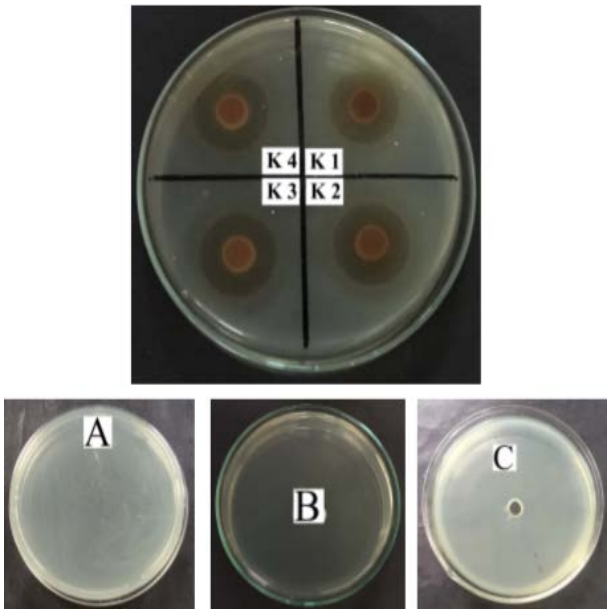

Figure 2: Inhibition zones of extract combination design. (A) positive control; (B) Negative control; (C) DMSO control; K1, K2,K3 and K4: combination design

One way ANAVA was conducted to determine the increasing effect of the extract concentration in combination design to the diameter of the inhibition zones. Following by Tukey's test to analyze the differences of each extract concentration in ability to inhibit the growth of *S. dysenteriae* bacteria. The ANAVA test results were performed in table 6 and the Tukey's result were in table 7.

Table 6: ANAVA Test Result

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.587	3	2.196	21.596	0
Intercept	5292.0	1	5292.0	52052.459	0
Concentration	6.587	3	2.196	21.596	0
Error	0.813	8	0.102		
Total	5299.4	12			
Corrected Total	7.4	11			

Note: H0 means there was no effect of the concentration increasing from the extract combination on the inhibitory diameter;

H1 : there was an effect of the concentration increasing from the extract combination on the inhibitory diameter

Table 7: Tukey Test Result

Combination	N	Subset	
		1	2
1	3	19.7333	
2	3		21.2667
3	3		21.4
4	3		21.6
Sig.		1	0.599

Note: Values in different columns showed significant differences at α = 0.05

In table 6, it was known that the value of sig = 0 or smaller than the real level (α = 0.05). This showed that there was an effect of concentration increasing of the combination extract on the inhibitory diameter. Whereas, based on the results of the Tukey test, it could be emphasized that the extract of combination 1 has a significant difference in the diameter of inhibition against other combinations. However, the combination of 2, 3 and 4 did not have a significant difference in inhibitory diameters from each other, only has a significant difference to the extract of combination 1 only. This showed that the other combinations had the same effect on inhibiting *S. dysenteriae*. This analysis was correlated with the antibacterial category of all combination design. Only combination design No. 1 was categorized as a moderate antibacterial candidate. While the others combination were grouped as strong antibacterial candidates. Furthermore, to find out whether there was a significant effect in inhibiting *S. dysenteriae* between a combination of fruit and pseudostem extract with the fruit banana ethanol extract alone was carried out by T-test. The results of the T test can be seen in Table 8.

Table 8: T Test Result

	t-Test for Equality of Means				
	t	df	Sig, (2-tailed)	Mean Difference	Std. Error Difference
Capability	Equal variances assumed 10,561	22	0	5,61667	0,5138

Note: H0 : no significant different; H1 : significant different

Based on Table 8, the results of the T test showed that there was a significant difference between the extract combination and single extract of fruit klutuk banana in inhibiting the growth of *S. dysenteriae*. This could be seen from the sig value. which was smaller than the real level (α = 0.05) which means the initial hypothesis was rejected.

Potassium Levels in A Combination of Extracts

The sample was prepared the same as the preparation for a single potassium content test. The absorbance of potassium standard solution with a concentration of 100, 200, 300, 400 and 500 ppm was measured to obtain a linear regression equation which of y = 0.00125 x + 0.0000317 with r = 0.996711. The potassium content data can be seen in Table 9.

Table 9: Potassium Level of Extract Combination Design

Combination	Potassium Level (%)		
	Fruit extract	Pseudostem Extract	combination
1	0.476	1.159	1.635
2	0.321	1.237	1.558
3	0.183	1.310	1.493
4	0.051	1.378	1.429

Based on the literature, the content of potassium in the oral rehydration solution (ORS) on the market is 0.15 g / 100 ml (1.5 g / L) or equivalent to 1.5%. The potassium content in the combination extract number 3 was closely similar to the content of potassium in ORS, which was 0.1493 g. It can be concluded that the extract combination design number 3 was a combination that can be used in dealing with cases of mild dehydration.

CONCLUSION

This study confirmed that the extract combination between the fruit and pseudostem ethanolic extract of Klutuk banana showed their potential efficacy as a synergist and natural antibiotic agents which improving the anti-dysentery activity and as a natural anti-dehydration agent that supply potassium in complying concentration with potassium content in the ORS commercial.

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