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by Mardiyanto Mardiyanto

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Nanoparticulate formulatiom of christmas palm seed (*Adonidia merrillii*) ethanolic extract containing lactic acid for antidiarrheal therapy

Nanoparticulate formulatiom of christmas palm seed (*Adonidia merrillii*) ethanolic extract containing lactic acid for antidiarrheal therapy

Mardiyanto[1a], Sofia Sandi[2b], Herlina[1c], Ivan Kurniawan[1d]

[1] Department of Pharmacy Study Program Faculty of Mathematics and Natural Sciences, University of Sriwijaya, Indonesia
[2] Depatment of Animal Science Faculty of Agriculture, University of Sriwijaya, Indonesia

[a] mardiyantounsri@gmail.com; [b] sofiasandi@yahoo.com, [c] rinaafdil@yahoo.com, [d] ivankurniawan@yahoo.com

Abstract: Preparation of Christmas Palm seeds ethanolic extract and lactic acid loaded nanoparticles aims to obtain an antidiarrheal (caused by *Escherichia coli*) therapy by using nano herbal. Preparation of the nanoparticles was done by ionic gelation method. There were three formulas (F) which had been evaluated in this study based on the difference amount of $Ca(OH)_2$, 12.5 mg for F1, 17.5 mg for F2, and 22.5 mg for F3. All formulas revealed an entrapment efficiency percentage of 75.489 over or less 0.563 (F1); 76.885 over or less 0.046 (F2); and 74.844 over or less 0.724% (F3). The second formula (F2) produced the highest %EE therefore it was distinguished as the optimum formula. The results of nanoparticles characterization of an optimum formula such as average diameter, poly dispersity index (PDI), and zeta potential using particle size analyzer were 1230.1 nm; 0.482; dan 27.5 mV respectively. An in-vivo antidiarrheal evaluation on rats using an optimum formula containing 1 gram of extract showed that nanoparticles possed an equal antidiarrheal effect to intraperitoneal gentamicin sulfate 8 mg/kg body weight with diarrhea inhibitory of 71.539%.

1. Introduction

Diarrhea is a bodily condition which signed by excretion of feces more than 3 times a day. Most case, diarrhea is caused by dehydration from the loss of fluid within the body through feces [1]. Infection usually involves diarrhea within gastrointestinal system by microorganism such as bacteria, viruses, and parasites. Initial infection is trigered by ingesting food or beverages which are contaminated with fecal matter through gastrointestinal tract or through direct infection from infected individual [2]. Diarrhea treatment includes replacement of loss fluid such as: electrolyte solution to prevent dehydration, regular intake of nutrition from food for gastrointestinal function recovery, antibiotic treatment, and the use of probiotic to alleviate simptoms. The use of probiotic in individual with diarrhea leads to the lower chances of diarrheal simptoms for presisting more than 4 days [3,4].

Christmass Palm (*Adonidia merrillii* (Becc.) Becc.) contains secondary metabolite compounds such as alkaloid, tanin, terpenoid, saponin, and flavonoid. Flavonoid compound within christmass palm had been proven as an antibacterial against *Staphylococcus aureus* and and *Escherichia coli* with minimum inhibitory concentration of 2% [5]. This antibacterial activity of the christmass palm has an increasing potential while combined with isolate lactic acid of probiotic bacteria.

Probiotic bacteria is a non pathogenic bacteria which have mutualistic symbiotism with its host by helping the digestion of its host food and modulizing host immunity system [6]. Probiotic bacteria can produce organic acid such as lactic acid (57.12 ppm), citric acid (139.93 ppm) andacetic acid (586.17 ppm) that can be metabolized by gut normal flora for balacing of normal flora within the gut by increasing its number within the intestine of individual [7].

Because of an antidiarrhea potensial possess by both of christmass palm seeds and isolate lactic acid, scientific experiment need to be done to prove both of these natural ingredients as an antidiarrheal drug in nanoparticles. The experiment use the male wistar rat induced with *E. coli* with OD of 0.3 at 580 nm.

2. Materials and Methods

2.1. Preparation of Adonidia merrillii (Becc.) Becc. Seeds extract

Fresh seeds of *Adonidia merrillii* was cleaned with flowing water then dried under ambient condition [8]. The dried seed then was grounded into powder and then 500 gram of seed powder was extracted with 1 L of 96% ethanol using maceration method for 48 hours. The macerate then was filtrated with filter paper and the residue was remacerated with 1 L of ethanol 96%. The filtrate was obtained from filtration then evaporated with temperature of 60^{o}C with *rotary evaporator*[8]. The yield of evaporated extract was obtained from previous step then calculated with formula below

$$\% \text{ yield} = \frac{weig\,ht\ of\ \ eveporated\ \ \ extract}{weig\,ht\ of\ seed\ powder} \times 100\% \quad (1)$$

2.2. Identification of flavonoids

The identification of flavonoids was done by reacting the extract of seed powder macerate with Shinoda reactant (solid Mg + HCl). The sample was then added with few drops of concentrated HCl then the magnesium was added as much as 0.20 mg. The macerate contains flavonoids while the solution turn into red in coloration [9].

2.3. Preparation of nanoparticles

Three formulas were formatted as shown in Table.1. Preparation was first done by preparing the solution of 105 mg chitosan within 21 mL of 50 mg/ml citric acid inside beaker glass and stirred with magnetic stirrer at speed of 75 rpm for 30 minutes and then 7 mL of the solution [10] for each formula was taken as M1. Natrium alginate as much as 150 mg was dissolved in 30 mL of API in beaker glass and homogenized using magnetic stirrer at 75 rpm for 30 minutes then each 10 mL of the solution was taken as M2 [11]. As much as 10 mg $CaCl_2$ was dissolved in 10 mL of aqua pro injection and then 2.5 mL of this solution was taken for each formula. $CaCl_2$ solution was then added 10 mg of lactic acid isolate that each consist of 1 mL of lactic acid of bacteria silage with 12.5 mg, 17.5 mg and 22.5 mg of $Ca(OH)_2$. The formation of nanoparticle was done by adding M2 solution drop by drop into M1 solution while mixing of magnetic stirrer and then to this solution the $CaCl_2$ solution and lactic acid bacteria isolate was added drop by drop until nanoparticles were formed.

Tabel 1. Nanoparticle Formula

Component	F1	F2	F3
Ethanol extract (g)	1	1	1
Chitosan (mg)	35	35	35
Natriumalginate(mg)	50	50	50
$CaCl_2$ 0,018M (mL)	2,5	2,5	2,5
Isolat BAL (ml)	1	1	1
$Ca(OH)_2$ (mg)	12,5	17,5	22,5

2.4. Particle Purification and %EE Determination (Entrapment Efficiency Percentage)

Particle purification was done by separating the particle and filtrate of 10 mL of nanoparticle solution using Vivaspin® 300 kDa and sentrifugated for 15 minutes [11]. The particle traped by the vivaspin then was added 30 mL of API and this solution then was reseparated for 3 times to obtain pure filtrate. Determination of %EE was done by making quercetin calibration curve of 0.002; 0.004; 0.006; 0.008;dan 0.01 mg/ml from 1 mg/ml quercetin in 96% ethanol solution, then the absorbance of each consentration of solution was measured using UV-Vis spectrophotometer at 371.8 nm. Absorbance measurement of nanoparticle filtrate was also done at 371.8 nm and %EE was calculated using formula below.

$$\%EE = \frac{\sum quercetin \ \ in\ extract - \sum quercetin \ \ in\ filtrate}{\sum quercetin \ \ in\ extract} \times 100\% \quad (2)$$

2.5. Particle characterization

Particle characterization include things such as diameter, distribution, dan particle zeta pontensial using *particle size analyzer* (PSA) through *dynamic light scaterring* (DLS) method. As much as 50 µl of purified Nanoparticle dispersion were obtained then diluted for 100 times with aquadest and 50 µL of this solution was analyzed using PSA [12,13].

2.6. Diarrhea Induction

E. coli from primary colony were inoculated into 20 ml of *nutrient brothc* and incubated at 37 ^{o}C until the optical density of the bacterial suspension was 0.3 at 580 nm [13]. The suspension of 0.5 mL then was given oraly to all of test subject [14].

2.7. Antidiarrheal Activity Assay

The test subject that have the sign of diarrhea then given the experiment procedure for 3 days.Then the subject was placed in 40 x 30 cm cage with each test group contain 6 rats.

Table 2. Classification of Group

Group	Criteria
Control +	Inj gentamisin sulfat 8 mg/kgBW
Control -	Exipient of formula
Extract	Extract 100 mg/kgBW
Nanoparticles	Formula optimum

2.8. Diarrhea Recovery Observation

The group of observation was showed in Table.2. Observation was done from day 1 to day 3 of experiment until the diarrhea was visibly stop or there was no longer any excess fluid in the feces [14].

The indicators observed for diarrhea recovery include body weight change percentage, inhibitory percentage, feces fluid diameter and the frequency of diarrhea. Diarrhea inhibitory percentage can be calculated using:

$$\%\ \text{inhibitory} = \left| \frac{(a-b)}{a} \right| \times 100\ \% \quad (3)$$

Abreviation:
a = average body weight change percentage in negative control group
b = average body weight change percentage in test group.

2.9. Data Analysis

Analysis done to see if there was any diarrhea recavery effect, the data were analized using ANOVA (*Analysis Of Variant*), with α 0,05 or 5%. If there is a difference than the data were analized using LSD (*Least Significant Different*).

3. Results

The result of the maceration was the extract had the yield of 29.28 g from 500 g seed powder and the yield percentage was 5.86%. The yield percentage was still inside the acceptable range for evaporated extract which mean the extraction process was effective in covering the metabolite out of the dried seed powder.

The test done was for the identification of flavonoid because it was the main metabolite that has the role of antibacterial in the formula used and has antidiarrheal activity. Magnesium and HCl react with flavonoid compound through reduction in benzopirone ring inside flavonoid structure which rises the red coloration from the conformation of auxochrome's flavonoid.

The regression curve was obtained as y = 0,0622x + 0,0367with r value of 0.998. The %EE value was obtained from the each formulacan was showed in Table 3.

Table 3. Percentage of Encapsulation Efficiency

Formula	**%EE + SD**
F1	75.4892 ± 0.5638
F2	76.8853 ± 0.0465
F3	74.8448 ± 0.7249

The result of diameter and particle distribution of the optimum formula was 1230.1 nm. Optimum formula showed that the result was neares-range of the nanoparticle size which 200 – 900 nm this was cause by one crucial parameter in the making of nanoparticle which was the homogenation process of the optimum formula.

In this experiment the antidiarrheal assay of christmass palm seed extract and lactic acid nanoparticle was done to see the diarrhea recovery of the nanoparticle on test subject of male wistar rat. The parameter observed in this experiment includes % inhibitory diarrhea, feces fluid diameter, and diarrhea frequencies [14]. Antidiarrheal assay experiment was done using gentamicin sulfate as positive control, gentamicin was chosen because of the sensitivity toward gram negative bacteria such as *E.coli*.

Table 4. Result of Antidiarheal Test

Group	Mean of BW day- (g)		Mean of % changes BW day- (g)		
	0	3	1	2	3
Control -	151.612	124.985	-11.1513	-12.903	-17.542
Control +	167.831	175.61	-2.921	-2.769	4.939
Extract	167.648	157.082	-7.659	-7.245	-5.021
Nanoparticles	166.363	168.248	-1.309	-0.271	1.449

Antidiarrheal test results form the value of the percent change in weight (Table.4) indicated the activity of the antidiarrheal nanoparticles extract was greater than the positive control group and extract. Based on the results of test data diameter liquid stool on the attachment 17 obtained all the test group experienced a decrease in diameter of liquid stool frequency and diarrhea on the second day except in the negative control group. Test result data showed a large group of nanoparticle preparation had liquid stool diameter and frequency of diarrhea the most small compared with other groups. This was because on the positive control group and only extract contains compounds which were anti-bacterial i.e. gentamicin and flavonoids which worked as an antibacterial by forming a bond of phenyl on the cell walls of the bacteria *E. coli* and interfere with the structure.

Tabel 5. Inhibition Percentage of Diarheal

Treatment	Percent of diare inhibition (%)
Negative	0
Positive	63,08403
Extract	36,63361
Nanoparticle	71,53956

Based on the percent inhibition of each group was obtained by a group of test material of ethanol extract seeds carrier nanoparticles Palm seed and isolates of lactic acid inhibited diarrhea of 71.539% while the percent inhibition of extracts of 36.634% in Table.5. This showed that an increase in the activity of the preparation nanoparticle carrier Antidiarrhoeal extracts and isolates of lactic acid bacteria. Increasing the activity due to the ability to penetrate the spaces between the cells and the cell walls of bacteria are better on nanoparticle preparations [21].

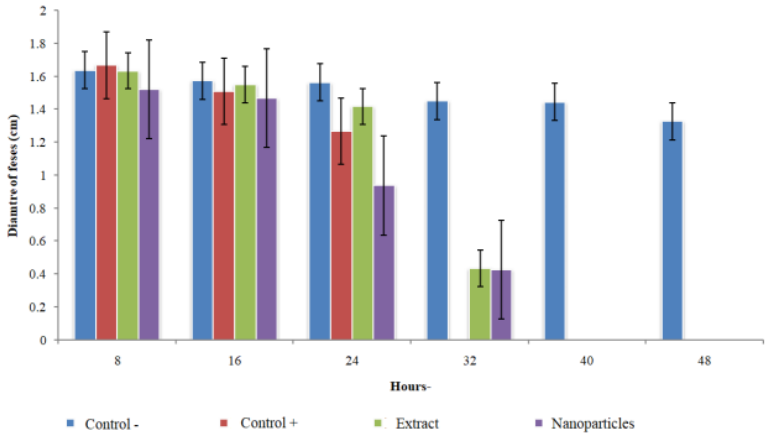
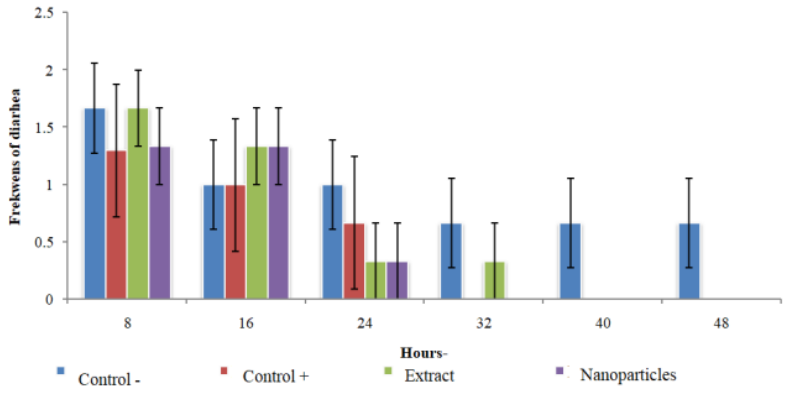


Figure 1. Mean diameter of fecal fluid

The results obtained % inhibition diarrhea of positive group larger than the percent inhibition of test groups in the form of extracts day-14. Antidiarrheal activity test results were showed in Table.5. The Shapiro Wilk normality test toward diarrhea healing showed that the data was normaly distributed ($p<0.05$). Statistical data of fecal fluid diameter showed that the data wasn't significantly different ($p>0.05$) between the positive control group, treatment group I, group II and group treatment treatment III. This shows that the Palm seed extract daughter has diarrhea that healing activity wass almost proportional to the positive control gentamicin sulfate injection in the healing process of diarrhea after 3 days. However, there were significant differences with the negative control group ($p<0.05$) on second and third day.



Figue 2. Frequency of diarehea of animal-tested

4. Discussion

Formulation revealed the optimum formula which was abbreviated by F2. The F2 can be seen had the highest %EE caused by high concentration of calcium ion that act as crosslinker reach optimum

concentration which caused the shrinkage of the particle did not happen as much as F3 and because of this reason the F2 was chosen as optimum formula [15].

The mixing of the polymer with only using magnetic stirrer instead of using sonicator probe affect the size of the particle because there was not enough kinetic energy to make the particle smaller[17]. The particle distribution or PDI using PSA for the optimum formula was 0.482 that still inside the range of fine distribution which is below 1 and that meaned the optimum formula was monodispersed [16]. Zeta potential value was obtained of 27.5 mV which meaned the particle of the optimum formula has enough charge to repel each other and prevent aggragation.

Gentamicin has ability to cure diarrhea was obtain from its mechanism as antibiotic. The induction process was done by orally injecting the rat of each group with suspension of *E.coli* as much as 5 mL/kg body weight [14]. Then the rats were put in observation cage that had been layered with filter paper at the bottom. The observation then was done from day-0 until day-3 of experiment and for the diarrhea frequency and feces fluid diameter were measured every 8 hours.

Based on the data obtained can be seen that the control treatment group had negative weight losspercentage because most of this group were not given drugs to inhibit the diarrhea. All treatment had an increased weight after the first day except for the negative control group continued to experience weight loss. A group of animal treatment nanoparticles look experience increased body weight greater than the positive control group and gentamicin groups extract. This is because the nanoparticle preparations not only contain compounds that are antibacterial i.e. Palm seed extract but also contain isolates of lactic acid that can be metabolized by intestinal flora into compounds that can be decrease motility of the gut and modulate the bodys prptection while the system extracts and gentamicin only work by inhibited the growth of bacteria caused diarrhea without modulated the bodys protection system [17,18].

The value of the percent change in a high weight on nanoparticle induced by two synergistic mechanisms of active substances which were used namely ethanol extracts of Palm seed and isolates of lactic acid. Extract works as a result of the antibacterial compound content of flavonoids that work forms a bond of phenol with membrane proteins to the surface of the bacterial cell wall and thus interfere with the structural function of the cell wall and cause material out of the intracellular cell. Isolates of lactic acid working as an antidiarrheal with normal flora of the gastrointestinal tract triggers for isolates of lactic acid bacteria metabolizes into the compound the butyric that can decrease bowel motility and modulate the immune system [16,18].

Component of nanoparticles in addition containing extracts which act as antibacterial and also isolates of lactic acid that can be metabolized by normal intestinal flora into compounds leads to stimulation of the formation of host defense peptides and butyric compounds that lower bowel motility so liquid stool can be reabsorbed by the body and decrease the frequency of diarhea. The results obtained by the test group had the diameter of semi-liquid stool in low frequency and smallest diameter compared to of all groups [19,20]. Quantitative measuring in Figure of average diameter of liquid stool and graph the frequency of diarrhea can be seen in Figure 1 and Figure 2. On the starting day was not indicated a significant therefore, the weight changes of the day was not so significant. However, the bacteria undergo phased of growth and started producing toxin causes diarrhea on day 1 so that the weight was significant changes. The difference in diarrhea healing with negative control occurs due to the absence of active compounds that can help the healing process of diarrhea.

Acknowledgements
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The test subject that have the sign of diarrhea then given the experiment procedure for 3 days.Then the subject was placed in 40 x 30 cm cage with each test group contain 6 rats.

Diarrhea Recovery Observation
The group of observation was showed in Table.2. Observation was done from day 1 to day 3 of experiment until the diarrhea was visibly stop or there was no longer any excess fluid in the feces [14]. The indicators observed for diarrhea recovery include body weight change percentage, inhibitory percentage, feces fluid diameter and the frequency of diarrhea. Diarrhea inhibitory percentage can be calculated using:
a = average body weight change percentage in negative control group
b = average body weight change percentage in test group.

Data Analysis
Analysis done to see if there was any diarrhea recovery effect, the data were analized using ANOVA (*Analysis Of Variant*), with α 0,05 or 5%. If there is a difference than the data were analized using LSD (*Least Significant Different*).

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The result of the maceration was the extract had the yield of 29.28 g from 500 g seed powder and the yield percentage was 5.86%. The yield percentage was still inside the acceptable range for evaporated extract which mean the extraction process was effective in covering the metabolite out of the dried seed powder.

The test done was for the identification of flavonoid because it was the main metabolite that has the role of antibacterial in the formula used and has antidiarrheal activity. Magnesium and HCl react with flavonoid compound through reduction in benzopirone ring inside flavonoid structure which rises the red coloration from the conformation of auxochrome's flavonoid.

The regression curve was obtained as y = 0,0622x + 0,0367with r value of 0.998. The %EE value was obtained from the each formulacan was showed in Table 3.

The result of diameter and particle distribution of the optimum formula was 1230.1 nm. Optimum formula showed that the result was neares-range of the nanoparticle size which 200 – 900 nm this was cause by one crucial parameter in the making of nanoparticle which was the homogenation process of the optimum formula.

In this experiment the antidiarrheal assay of christmass palm seed extract and lactic acid nanoparticle was done to see the diarrhea recovery of the nanoparticle on test subject of male wistar rat. The parameter observed in this experiment includes % inhibitory diarrhea, feces fluid diameter, and diarrhea frequencies [14]. Antidiarrheal assay experiment was done using gentamicin sulfate as positive control, gentamicin was chosen because of the sensitivity toward gram negative bacteria such as *E.coli*.

Antidiarrheal test results form the value of the percent change in weight (Table.4) indicated the activity of the antidiarrheal nanoparticles extract was greater than the positive control group and extract. Based on the results of test data diameter liquid stool on the attachment 17 obtained all the test group experienced a decrease in diameter of liquid stool frequency and diarrhea on the second day except in the negative control group. Test result data showed a large group of nanoparticle preparation had liquid stool diameter and frequency of diarrhea the most small compared with other groups. This was because on the positive control group and only extract contains compounds which were anti-bacterial i.e. gentamicin and flavonoids which worked as an antibacterial by forming a bond of phenyl on the cell walls of the bacteria *E. coli* and interfere with the structure.

concentration which caused the shrinkage of the particle did not happen as much as F3 and because of this reason the F2 was chosen as optimum formula [15].

The mixing of the polymer with only using magnetic stirrer instead of using sonicator probe affect the size of the particle because there was not enough kinetic energy to make the particle smaller[17]. The particle distribution or PDI using PSA for the optimum formula was 0.482 that still inside the range of fine distribution which is below 1 and that meaned the optimum formula was monodispersed [16]. Zeta potential value was obtained of 27.5 mV which meaned the particle of the optimum formula has enough charge to repel each other and prevent aggragation.

Gentamicin has ability to cure diarrhea was obtain from its mechanism as antibiotic. The induction process was done by orally injecting the rat of each group with suspension of *E.coli* as much as 5 mL/kg body weight [14]. Then the rats were put in observation cage that had been layered with filter paper at the bottom. The observation then was done from day-0 until day-3 of experiment and for the diarrhea frequency and feces fluid diameter were measured every 8 hours.

Based on the data obtained can be seen that the control treatment group had negative weight losspercentage because most of this group were not given drugs to inhibit the diarrhea. All treatment had an increased weight after the first day except for the negative control group continued to experience weight loss. A group of animal treatment nanoparticles look experience increased body weight greater than the positive control group and gentamicin groups extract. This is because the nanoparticle preparations not only contain compounds that are antibacterial i.e. Palm seed extract but also contain isolates of lactic acid that can be metabolized by intestinal flora into compounds that can be decrease motility of the gut and modulate the bodys prptection while the system extracts and gentamicin only work by inhibited the growth of bacteria caused diarrhea without modulated the bodys protection system[17,18].

The value of the percent change in a high weight on nanoparticle induced by two synergistic mechanisms of active substances which were used namely ethanol extracts of Palm seed and isolates of lactic acid. Extract works as a result of the antibacterial compound content of flavonoids that work forms a bond of phenol with membrane proteins to the surface of the bacterial cell wall and thus interfere with the structural function of the cell wall and cause material out of the intracellular cell. Isolates of lactic acid working as an antidiarrheal with normal flora of the gastrointestinal tract triggers for isolates of lactic acid bacteria metabolizes into the compound the butyric that can decrease bowel motility and modulate the immune system[16,18].

Component of nanoparticles in addition containing extracts which act as antibacterial and also isolates of lactic acid that can be metabolized by normal intestinal flora into compounds leads to stimulation of the formation of host defense peptides and butyric compounds that lower bowel motility so liquid stool can be reabsorbed by the body and decrease the frequency of diarhea. The results obtained by the test group had the diameter of semi-liquid stool in low frequency and smallest diameter compared to of all groups [19,20]. Quantitative measuring in Figure of average diameter of liquid stool and graph the frequency of diarrhea can be seen in Figure 1 and Figure 2. On the starting day was not indicated a significant therefore, the weight changes of the day was not so significant. However, the bacteria undergo phased of growth and started producing toxin causes diarrhea on day 1 so that the weight was significant changes. The difference in diarrhea healing with negative control occurs due to the absence of active compounds that can help the healing process of diarrhea.

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LEMBAR
HASIL PENILAIAN SEJAWAT SEBIDANG ATAU *PEER REVIEW*
KARYA ILMIAH: JURNAL ILMIAH

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Penilai I

Prof. Aldes Lesbani, PhD
NIP. 197408121998021001
Unit Kerja: Jurusan Kimia
Bidang Ilmu: Kimia Anorganik
Jabatan/Pangkat: Guru Besar

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Dr.rer.nat Risfidian Mohadi, MSi
NIP.19771127200501003
Unit Kerja: Kimia
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