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Formulation, Evaluation and Antibactery Activity Test of Gastroretentive Mucoadhesive Granul Matrix Earthworm Extract (*Lumbricus rubellus*)

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ABSTRAK

Ekstrak cacing tanah (*Lumbricus rubellus*) memiliki aktivitas antimikroba karena menghasilkan senyawa lumbricine dalam kadar yang tinggi namun mudah terdenaturasi di dalam lambung sehingga kelarutan dan aktivitas biologisnya berubah. Oleh karena itu aplikasinya dapat dikembangkan dalam bentuk sediaan mukoadhesif gastrorentetif. Tujuan dari penelitian ini adalah untuk mengoptimasi dan mengevaluasi aktivitas antibakteri sediaan mukoadhesif gastrorentetif matriks ekstrak cacing tanah (*Lumbricus rubellus*). Matriks dibuat dengan menggunakan teknik preparasi polimer mukoadhesif granulasi basah seperti kitosan, HPMC, dan natrium CMC dengan konsentrasi yang ditentukan berdasarkan desain kisi-kisi sederhana yang menghasilkan 7 formula granul. Formula granul yang diperoleh kemudian dievaluasi meliputi uji waktu alir dan sudut diam, uji distribusi ukuran partikel, uji kompresibilitas, uji disolusi, dan uji aktivitas antibakteri. Hasil penelitian menunjukkan bahwa laju alir pada formulasi granul F3 memenuhi persyaratan laju alir >10 g/s, hal ini disebabkan karena sudut diam pada formula F1-F5 memiliki sifat alir yang baik yaitu di bawah 30^o, berbeda dengan F6 dan F7 yang memiliki sudut diam 30-40^o yang mendukung sifat alir. Formula F1-F7 sebagian besar tertahan pada ayakan nomor mesh 20 dengan ukuran 426-850 μm, berdasarkan hasil distribusi ukuran partikel dengan menggunakan metode ayakan bertingkat, indeks kompresibilitas granul formula F1-F7 sebesar 10% dan hasil pengukuran pada menit ke-30 menunjukkan bahwa rata-rata disolusi granul F1-F3 adalah 97 77%, 97,77%, dan 101,22% sedangkan formula granul F4-F7 tidak menunjukkan pelepasan obat yang lambat karena pada menit ke-30 obat yang dilepaskan bervariasi dan uji aktivitas antibakteri granul Formula 1-Formula 7 secara berturut-turut adalah 6 mm, 5,5 mm, 8 mm, 3,5 mm, 4,7 mm, 2,1 mm, dan 3,3 mm Hasil evaluasi karakteristik sediaan granul gastroretentive mukoadhesif ekstrak cacing tanah menunjukkan bahwa sediaan tersebut memenuhi persyaratan sediaan granul dan menunjukkan sifat antibakteri. Di antara ketujuh formula yang diuji, formula 3 memiliki efektivitas aktivitas antibakteri yang paling tinggi.

Kata Kunci: Ekstrak Cacing Tanah, Mukoadhesif Gastroretentive, Evaluasi Granul, Aktivitas Antibakteri

ABSTRACT

Earthworm extract (*Lumbricus rubellus*) has antimicrobial activity because it produces high levels of the lumbricine compound but is easily denatured in the stomach so that its solubility and biological activity change. Therefore its Application can be developed in the form of gastrorentetif mucoadhesive preparation. The purpose of this study was to optimize and evaluate the antibacterial activity of gastroretentive mucoadhesive preparation matrix of earthworm extract (*Lumbricus rubellus*). The matrix utilized a preparatory technique of applying wet granulation mucoadhesive polymers such as chitosan, HPMC, and sodium CMC with concentrations determined based on simple lattice design resulting in 7 granule formulas. The granule formulas obtained were then evaluated including flow time and dwell angle tests, particle size distribution tests, compressibility tests, dissolution tests, and antibacterial activity tests. The results showed that the flow rate on the F3 granule formulation met the requirements of flow rate >10 g/s, a consequence of measuring the angle of repose in the F1-F5 formula had good flowability which was below 30^o, in contrast to F6 and F7 which had an angle of repose of 30-40^o favorable to flowability. The F1-F7 formula was mostly preserved in mesh number 20 sieve with a size of 426-850 μm, according to the results of particle size distribution utilizing the multistage sieve method, the compressibility index of the F1-F7 granule formula was 10% and the measurement results at the 30 minute showed that the average dissolution of the F1-F3 granules was 97.77%, 97.77%, and 101.22% respectively while the F4-F7 granule formulas did not show slow drug release because at the 30th minute the drug released ranged and the antibacterial activity test of Formula 1-Formula 7 granules is respectively 6 mm, 5.5 mm, 8 mm, 3.5 mm, 4.7 mm, 2.1 mm and 3.3 mm. The results of the evaluation of the characteristics of earthworm extract mucoadhesive gastroretentive granule preparation demonstrated that the preparation met the requirements for granule preparation and exhibited antibacterial properties. Among the seven formulas tested, formula 3 had the highest efficacy of antibacterial activity.

Keywords: Earthworm Extract, Gastro Retentive Mucoadhesive, Evaluation Of Granules, Antibacterial Activity

INTRODUCTION

Antibiotic resistance is a narrower term than antimicrobial resistance, as it only relates to resistance in drugs that kill bacteria. However, because most infections in society are due to bacteria, antibiotic resistance is now becoming more urgent to deal with. People who experience resistance to antibiotics will make the disease in their body difficult to heal and can even cause death. To overcome this, the way is to use natural alternative materials, because natural alternative materials have less side effects. One of the natural ingredients that we can use to inhibit the development of *Salmonella thypi* and *Helicobacter pylori* is earthworm extract (Anggun Vernanda et al., 2018).

Earthworms have been utilized as traditional medicinal ingredients in several places in Indonesia, the earthworm that is often used is *Lumbricus rubellus*. Earthworm meal (*Lumbriscus rubellus*) contains high protein equivalent to fish meal (55-60%) crude protein. Amino acid analysis shows that *Lumbriscus rubellus* contains essential and non-essential amino acids. Essential amino acids are dominated by isoleucine, lysine, and leucine. Non-essential amino acids are dominated by glutamate and serine. *Lumbriscus rulbellus* flour contains 65.63% crude protein content and proline amino acids about 15% of the total amino acids (Prasetyo et al., 2017).

Lumbricus rubellus contains bioactive lumbricin which has antimicrobial activity. Lumbricin is a broad-spectrum antimicrobial peptide that can inhibit gram-positive and negative bacteria (broad spectrum) and is positively charged (Fauzi et al., 2022). Positively charged peptides are known to directly affect macromolecular synthesis by causing cell wall depolarization damage. The mechanism of action of lumbricin is by causing changes in the membrane permeability mechanism so that the cell is lysed. However, protein is a molecule that is quite easily influenced by physical and chemical factors so it is easy to denature by stomach acid. In the event that the protein is denatured, its solubility and biological activity will change. The addition of salt to a solution containing protein can cause the protein to precipitate. Giving alcohol or heating will also cause the protein to clump. In addition to its thermolabile nature, protein is also amphoteric. At low pH, the amino group will react with H+ ions so that the protein will be positively charged at high pH conditions, the carboxylate group will react with OH- ions and cause the protein to be negatively charged thus causing the effectiveness of the protein to not be optimal, therefore the protein must be in a more neutral state so that the protein will not be denatured and can function optimally (Yuswadinata & Wathoni, 2021). These characteristics are a challenge in making pharmaceutical preparations with peptide and protein active ingredients, so their utilization can be developed in the form of gastro retentive mucoadhesive preparations.

To overcome such problems and to improve drug bioavailability, slowed drug delivery systems with extended residence time in the stomach can be used . Gastro retentive drug delivery systems (GRDDS) is a drug delivery system in which drug release occurs in the stomach for a longer period in a controlled manner to obtain optimal bioavailability (Moh Kahfi, 2012). Extended residence time of antimicrobial agents is desirable to provide more effective eradication (Pakki, 2016). Gastro retentive dosage forms (GRDFs) are designed to be retained in the stomach for an extended time and to release their active substances, thus allowing the drug to be retained and extended in the upper part of the gastrointestinal tract (Febryani, 2018).

Based on this description, it is interesting to research to optimize and evaluate the gastro retentive mucoadhesive preparation matrix of earthworm extract. The goal of this research is to identify the ideal formula and evaluation of the gastro retentive mucoadhesive preparation matrix of earthworm extract which is expected to optimize its therapeutic effect.

RESEARCH METHOD

Extraction

Earthworms (*Lumbricus rubellus*) that had been dried until their weight reached 800 grams were extracted by infusion at a

temperature of 50^{0}C for 15 minutes with water. The extract is then filtered through filter paper to produce a filtrate. Then it is concentrated using a water bath to complete the evaporation process until a thick extract is obtained. (Fauzi et al., 2022; Magfirah, 2022).

Preparation of Mucoadhesive Gastroretentive Granule Formula of Earthworm Extract

Mucoadhesive gastro retentive granule preparation of earthworm extract will be made by wet granulation method (Table 1). The earthworm extract used in all formulas is 100 mg, then added with chitosan, HPMC, and sodium CMC according to the results of simple lattice design and also the addition of lactose in each formula. Then crushed until homogeneous and a mixed solvent of 96% alcohol and distilled water (1:1) until a fishable mass was formed. The mass obtained was then sieved using a 14 mesh sieve. Dried in an oven at 60^{o}C for 4 hours. The dried granules were sieved again using a sieve. The granules obtained were evaluated.

Table 1. Mucoadhesive Gastroretentive Granule Formula

No.	Materials	Formula (mg)						
		F1	F2	F3	F4	F5	F6	F7
1	Earthworm extract	100	100	100	100	100	100	100
2	Chitosan	40	60	70	80	90	100	110
3	HPMC	100	110	120	130	140	150	160
4	Na CMC	10	20	30	40	50	60	70
5	Avicel PH-102	Ad 500	Ad 500	Ad 500	Ad 500	Ad 500	Ad 500	Ad 500

Evaluation of Mucoadhesive Gastroretentive Formula Of Earthworm Extract Granul

1. Flow Time And Dwell Angle Test

The ability of the granule to flow can be seen from the number of granules that flow every second and from the angle of lying of the granule. Evaluation of the flow properties and angle of repose of the granule will then be taken into consideration in ensuring weight uniformity. The test of flow properties and angle of repose was carried out with a procedure that is as much as 10 g of granules inserted into the flow time test tool in the funnel. Then the bottom of the funnel is covered with paper, then open the funnel cover to flow the granule to be tested. Then record the granule flow time and measure the angle of repose of the granule. Granule Flow time can be calculated by the equation:

Flow time = $\frac{w}{v}$ g/s

The dwell angle test can be calculated by the equation :

Dwell Angle test = $\tan^{-1}\frac{h}{r}$

2. Particle Size Distribution Test

Determination of particle size distribution is carried out by the multilevel sieve method by arranging the sieve from the largest to the smallest sieve number for 10 minutes. Then weighed the granules that were stuck in each mesh sieve number.

3. Compressibility Test

Weigh 100 grams of granules and then put them in a measuring cup with an initial volume of 210 mL. Then put the granule into a beaker glass and compress the granule 500 times with a test tool, after being given a knock 500 times record the final volume results.

4. Dissolution Test

The dissolution test was carried out using a USP type II chamber (paddle) or paddle stirrer for 6 hours with an HCl medium pH of 3.0 which describes the pH of the stomach when filled with food. The dissolution test was carried out by entering the granule into the chamber and then covering it with a sieve so that the granule remained at the bottom of the chamber. The temperature was set to a constant 37^{0}C±0,5^{0}C. The media used was HCl pH 3.0 as much as 900 ml, with a stirrer rotation of 50 rpm.

Sampling of the solution was carried out at the 5th minute, 10, 20, 30, 40, 50 and 60 for 5.0 ml each. Then 5 ml of HCl pH 3 replacement liquid was added as a correction factor. The samples obtained were measured for absorbance with a UV spectrophotometer at a wavelength of 650 nm.

Antibacterial Activity Test

The antibacterial activity test used the well diffusion method Nutrient Agar (NA) media of as much as 25 ml was mixed with 25 µL of test bacterial suspension (staphylococcus aureus), poured into a sterile petri dish and left until it solidified, made wells with a diameter of ± 6 mm using a Cork Borer. Each cup contains 4 holes or wells, the first hole for negative control in the form of 10% DMSO solvent as much as 30 µL, the second hole for positive control in the form of ciprofloxacin antibiotic as much as 30 µL and 2 holes for extracts and nanoparticle preparations each as much as 30 µL, then incubated for 24 hours at 37 ° C, then observed and measured the diameter of the inhibition zone with a caliper.

RESULTS AND DISCUSSION

In this study, 7 formulas of earthworm extract mucoadhesive gastro retentive granules were made. Formulas F1, F2, F3, F4, F5, F6, and F7 were made using a combination of chitosan-HPMC- sodium CMC. All formulas were made using the wet granulation method. The wet granulation method is used because it produces granules that are more compact and easier to manufacture. On organoleptic observation of the granules, a brown color was obtained, with a distinctive aroma with an irregular shape and no taste in all formulas.

In the granule flow rate test results, the granule data obtained in formula F1, F2, F3, F4, F5, F6, F7, namely, 9.52±0.06; 9.36±0.53; 10.22±0.16; 5.27±0.44; 5.11±, 4.43±0.40; 3.32±0.40 g/s. Flow time is the time required for the granule to flow. The ability of granules to flow can be seen from the number of granules that flow every second. A good granule is a granule that can flow freely, said to be free if the flow rate is >10 g/s (Febriyani *et a.,l* 2018). Of the seven formulas, the formula closest to the good granule category is formula F3 with a value of 10.22, while granules that have poor flowability are formula F7. Formula F7 with a flow rate value of 3.32. This happens because the high concentration of NaCMC makes the granule flow rate decrease due to the formation of agglomerates. The results of the calculation of the granule flow rate can be seen in Table 2.

Table 2. The results of granule flow rate

No.	Formulas	Replicate			Average ±SD
		1	2	3	
1	F1	9.55	9.56	9.45	9.52±0.06
2	F2	9.25	8.90	9.95	9.36±0.53
3	F3	10.11	10.34	9,90	10.22±0.16
4	F4	5.67	4.80	5.35	5.27±0.44
5	F5	4.90	4.98	5.45	5.11±0.29
6	F6	4.00	4.80	4.50	4.43±0.40
7	F7	3.38	3.68	2.90	3.32±0.40

The results of the Dwell angle test of the granule, obtained data on formulas F1-F7 respectively, namely, 29.98±0.83; 28.22±0.16 ; 29.36±0.92; 28.60±0.23; 29.11±0.78; 32.12±0.62; 32.39±0.29°. Dwell angle is the angle formed by the granule at maximum on a horizontal surface (Febriyani *et al*, 2018). The angle of repose value is inversely proportional to the flow rate, the smaller the angle of repose, the greater the flow rate. If the Dwell angle obtained is less than 30°, it indicates that the granule can flow freely, while granules whose values are greater than or equal to 40° are usually less able to flow well (Febriyani *et al*, 2018). Of the seven formulas, F1 and F2 are formulas that meet the requirements of a good granule, while formulas F3, F4, F5, F6, and F7 obtain values above 30° but are still in the good category. The size of the angle formed

by the granule is influenced by the size of the particles, the magnitude of the tensile force, and the frictional force of the particles. The smaller the particle size, the higher the cohesive force, the high cohesive force causes the granule to be difficult to flow and causes the angle of rest obtained to be greater (Febriyani *et al*, 2018). The results of the Dwell angle of the granule can be seen in Table 3.

Table 3. The Results Dwell Angle Of Granule

No.	Formulas	Replicate			Average ±SD
		1	2	3	
1	F1	29.95	28.56	28.45	29.98±0.83
2	F2	28.11	28.34	28,85	28.22±0.16
3	F3	29.36	28.40	29.45	29.36±0.92
4	F4	28.67	28.80	28.35	28.60±0.23
5	F5	28.90	29.98	28.45	29.11±0.78
6	F6	32.00	32.80	31.56	32.12±0.62
7	F7	32.38	32.68	32.10	32.39±0.29

In testing particle size distribution, the data obtained in all nine formulas have a particle size distribution with a vulnerable particle size of 426-850 µm or mesh size 20 (Febriyani *et al*., 2018). This happens because the granulation process is carried out using a mesh 20 sieve so that the distribution of granule particle size is more retained on mesh sieve 20 with a particle size ranging from 426-850 µm. The percentages of granules with sizes 426-850 µm in formulas F1-F7, respectively, are 45.8; 68.5; 58.7; 67.6;69.5; 68.8 dan 58.5 %. Granule weight per mesh (%) can be seen in Figure 1.

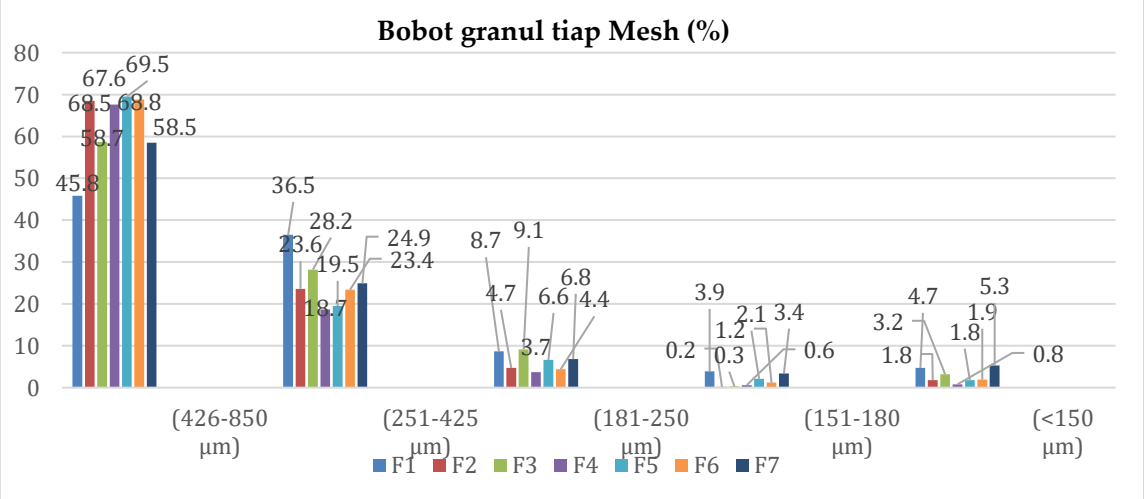

Figure 1. Granule Weight/Mesh (%)

Dissolution tests were conducted for all formulas using a type 2 dissolution device (paddle type) with a speed of 50 rpm. The medium used was 0.1 N HCl solution because the in vitro tests carried out were adjusted to the pH at which the drug was expected to be released with the medium temperature maintained at 37 ± 0.5° C, adjusted to human body temperature. To calculate the amount of earthworm extract dissolved, a standard curve of Lumbricin in the dissolution medium used was made. Lumbricin solution made in the dissolution medium gives a maximum wavelength value of 700 nm. The standard curve equation obtained is y = 0.0521 + 0.0117 with a value of r = 0.995. The dissolution test results showed that of the seven formulas made, there were only three formulas that showed slow drug release (namely FI, F2, and F3), while the other four formulas (namely F4, F5, F6, and F7) did not show slow drug release because at minute 40 the drug released ranged from 94% -

101%, where in that concentration range the drug was almost completely released or even completely released.

The three formulas that showed slow drug release (i.e. FI, F2, and F3), were formulas prepared using 100 mg, 110 mg, and 120 mg HPMC respectively. Formula I released 25.22% ±0.00047 of the drug at 20 minutes and 97.77% ±0.00017 at 60 minutes. Formula 2 released 21.76%±0.00037 of the drug at 20 minutes and 97.77% ±0.00027 at 60 minutes. Formula 3 released the drug as much as 18.31%±0.00017 at 20 minutes and 101.22% ±0.0007 at 60 minutes. Formula 3 is the formula that releases the drug the slowest compared to Formula 1 and Formula 2. Formula 4 released the drug as much as 7.94%±0.013 at 20 minutes and 101.22% ±0.0017 at 40 minutes. Formula 5 released the drug as much as 11.40%±0.00017 at 20 minutes and 97.77% ±0.023 at 40 minutes.

Formula 6 released the drug as much as 21.76%±0.00017 at 20 minutes and 94.31% ±0.039 at 40 minutes. Formula 7 released the drug as much as 39.04%±0.023 at 20 minutes and 101.22% ±0.041 at 40 minutes.. The dissolution results can be seen in Figure 2.

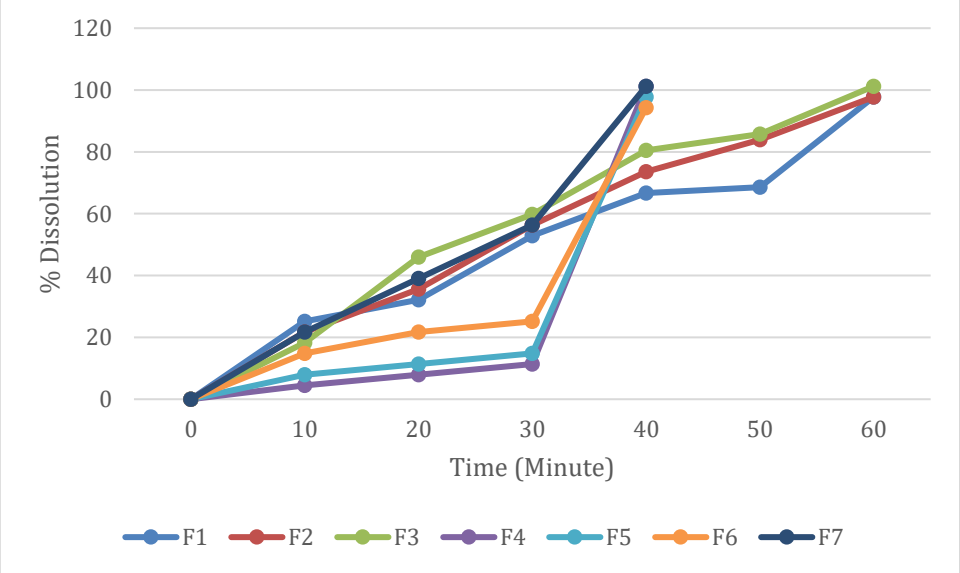

Figure 2. Graph of Dissolution Test Results Formula

Data from the observation of the diameter of the inhibition zone through measurement using a caliper after *Salmonella thyposa* was incubated for 1x24 hours at 37ºC are presented in Table 5. The measurement data of inhibition zone diameter (mm) against *Salmonella thyposa* F1-F7 bacteria were 6 mm; 5.5 mm; 8 mm, 3.5 mm; 4.7 mm; 2.1 mm, and 3.3 mm respectively.

Based on the research conducted, the results of various formulas in Table 4 are obtained, namely in Formula 4- formula 7 has an inhibitory power below 5 mm, while in formulas 1-3, it has an inhibitory power above 5 mm with formula 3 which has the closest inhibitory power to the positive control, which is 8 mm. The positive control results have a strong inhibition of 10 mm. The positive control used is the antibiotic chloramphenicol, chloramphenicol is an antibiotic that has a mechanism of inhibiting microbial cell protein synthesis and is still used as a treatment for typhoid fever because it is effective, cheap, easy to obtain, and can be given orally.

Earthworms contain an antibacterial called lumbricilin. Lumbricin is a peptide compound composed of complete amino acids, especially proline. The protein possessed by earthworms causes the formation of pores in the bacterial cell wall. This causes the cytoplasm of the bacterial cell to be exposed to the external environment, which can disrupt the activity in the bacterial cell and cause death, because what is damaged is the cell structure of the bacteria itself, making it more difficult to resist.

Table 4. Antibactery Activity Test Of Gastroretentive Mucoadhesive Of Earthworm Extract

No.	Sample	Inhibition zone			Average ±SD
		Replicate (1)	Replicate (2)	Replicate (3)	
1	Formula 1	6.5 mm	6 mm	5.5 mm	6 ±0.50
2	Formula 2	5 mm	5.5 mm	6 mm	5.5±0.50
3	Formula 3	7,5 mm	8 mm	8.5 mm	8±0,50
4	Formula 4	3.2 mm	3.5 mm	3.8 mm	3.5±0.30
5	Formula 5	4.5 mm	4.8 mm	5 mm	4.7±0.25
6	Formula 6	2.5 mm	2 mm	2 mm	2.1±0.28
7	Formula 7	3.8 mm	3.2 mm	3 mm	3.3±0.42
8	Control positive	10 mm	10 mm	10 mm	10±0

Based on the research results, the third formulation of the three components (chitosan, HPMC, and NaCMC) has higher levels than the first and second formulations, this is because The chitosan content in the third formulation is (70 mg), higher than the first (40 mg) and second formulations (60 mg). Chitosan acts as an active ingredient, the higher the level, the higher the effectiveness of the formulation. And the HPMC content in the third formulation is (120 mg), higher than the first (100 mg) and second formulations (110 mg).

HPMC acts as a thickening and matrix forming agent, the higher the level, the better the consistency and viscosity of the formulation. The NaCMC content in the third formulation is (30 mg), higher than the first (10 mg) and second (20 mg) formulations. NaCMC also acts as a thickening and matrix forming agent, the higher the level, the better the consistency of the formulation (Febriyani, *et al*.2018)

There are also formulations four to seven that are not good when compared to formulations one to three for several reasons: First, the fourth to seventh formulations contain HPMc at lower doses than formulations 1-3. Formulations 1-3 contain HPMc at doses of 100 mg, 110 mg, and 120 mg, while formulations four to seven contain HPMc at doses of 130 mg, 140 mg, 150 mg, and 160 mg. Increasing the HPMc dose from 100 mg to 120 mg can increase the effectiveness of the formulation, but increasing the dose from 120 mg to 130 mg and above will not significantly increase the effectiveness of the formulation. Second, the fourth to seventh formulations contain Nacmc at lower doses than formulations 1-3.

Formulations 1-3 contain Nacmc at doses of 40 mg, 50 mg and 60 mg, while formulations four to seven contain Nacmc at doses of 50 mg, 60 mg and 70 mg. Increasing the Nacmc dose from 40 mg to 50 mg and above will not significantly increase the effectiveness of the formulation. Third, the fourth to seventh formulations contain lower doses of chitosan than formulations 1-3. Formulations 1-3 contain chitosan at doses of 80 mg, 90 mg, and 100 mg, while formulations four to seven contain chitosan at doses of 100 mg, 110 mg, and 120 mg. Increasing the dose of chitosan from 80 mg to 100 mg can increase the effectiveness of the formulation, but increasing the dose from 100 mg to 110 mg and more will not increase the effectiveness of the formulation significantly. For this reason, the fourth through seventh formulations are not good compared to the formula. Therefore, the third formulation is the best formulation because it contains the highest levels of chitosan, HPMC and NaCMC. High levels of each component will increase the effectiveness, consistency and overall quality of the formulation (Febriyani, *et al*.2018).

From the results of research by Sugito, S., & Slamet, S. using the disc diffusion method and as a comparison control using chloramphenicol showed that earthworm extract (*Lumbricus rubellus*) for all concentrations, namely 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% effective in inhibiting the growth of Salmonella typhi. The largest diameter of the inhibition area was

obtained with a concentration of 100% by 20 mm at 24 hours incubation and a positive control of 23 mm while at a concentration of 90%-10% there was a decrease in the inhibition area, namely 90% by 19mm, 80% by 18mm, 70% by 17mm, 60% by 16mm, 50% by 15mm, 40% by 12mm, 30% by 10mm, 20% by 8mm, 10% by 6mm This means that earthworm extract (*Lumbricus rubellus)* is effective for inhibiting Salmonella typhi bacteria.

Based on previous research Sugito & Slamet, Nur Indah, and Syarifah allow earthworms (Lumbricus Rubellus) can be used as a natural alternative medicine in treating typhoid fever. It is expected that further researchers examine the antibacterial effect of earthworm extract (*Lumbricus rubellus*) against other types of bacteria and identify the active compounds that play the most antibacterial role in earthworm extract (*Lumbricus rubellus*) using different methods.

CONCLUSION

The findings of the research that has been done that, the best formula is formula 3 with the composition of Chitosan 70 mg, HPMC 120 mg and Na CMC 30 mg and Formula 3 provides the effectiveness of earthworm extract (Lumbricus rubellus) against Salmonella Typhi bacteria with an inhibition zone of 8 mm.

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